University of Alberta

LIBRARY RELEASE FORM

NAME OF AUTHOR: **CINDY QUN GAO**

TITLE OF THESIS: **MATRIX METALLOPROTEINASE-2 MEDIATES CYTOKINE-INDUCED MYOCARDIAL CONTRACTILE DYSFUNCTION**

DEGREE: **MASTER OF SCIENCE**

YEAR THIS DEGREE GRANTED: **2001**

University of Alberta

MATRIX METALLOPROTEINASE-2 MEDIATES CYTOKINE-INDUCED MYOCARDIAL CONTRACTILE DYSFUNCTION

BY

CINDY QUN GAO

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Pediatrics

Edmonton, Alberta, Canada

Fall, 2001

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **MATRIX METALLOPROTEINASE-2 MEDIATES CYTOKINE-INDUCED MYOCARDIAL CONTRACTILE DYSFUNCTION** submitted by **Cindy Qun Gao** in partial fulfillment of the requirement of the degree of Master of Science

ABSTRACT

Pro-inflammatory cytokines depress myocardial contractile function by enhancing myocardial peroxynitrite production. As oxidants like peroxynitrite are known to activate matrix metalloproteinases (MMPs), we examined whether changes in MMP activity contribute to cytokine-induced myocardial dysfunction. Isolated working rat hearts were perfused with pro-inflammatory cytokines in Krebs-Henseleit buffer for 120 minutes. Cytokines induced a marked decline in myocardial contractile function accompanied by enhanced tissue inducible nitric oxide synthase activity (iNOS) and increased perfusate dityrosine level (a marker of peroxynitrite). Perfusate pro-MMP-2 increased and was accompanied by a significant decrease in tissue pro-MMP-2 level. TIMP-4 protein expression decreased in cytokine-treated hearts. A peroxynitrite scavenger (FeTPPs), MMP inhibitors (Ro31-9790, PD16693), and an anti-MMP-2 antibody attenuated the decline in cytokine-induced myocardial dysfunction. These data indicate that functional depression in pro-inflammatory cytokine exposed hearts is mediated, in part, by MMP-2. Scavenging peroxynitrite or inhibiting MMP activity may be possible therapeutic approaches in treating inflammatory heart disease.

ACKNOWLEDGEMENTS

I express deepest gratitude to my supervisor, Dr. Richard Schulz, for his knowledgeable guidance, encouragement and patience during the course of this study. I consider myself very fortunate to have very close supervision from such an inquisitive explorer in the field of nitric oxide and matrix metalloproteinase. I also acknowledge his personal support, understanding and adjustment in my favor, which helped me through on various occasions.

My next appreciation goes to Dr. Gary Lopaschuk and Dr. Marek Radomski for their valuable suggestions, discussion, support, and encouragement as my supervisory committee members. Their helpful thoughts and recommendations are critical for the successful completion of this degree.

I thank Dr. Grzegorz Sawicki and Dr. Weijie Wang for their help on different occasions. I also appreciate technical help for perfusion from Donna Panas.

My gratitude also goes to Mary-Jo Boeglin, Lorraine Marler, and Judy Deuel who helped me through various administrative details in my program.

I am thankful to the Heart and Stroke Foundation of Alberta for the financial support.

Finally, I am grateful to my parents Xihua Gao and Huixiang Zhang, whose discipline and love gave me eyes to see the world. My deepest appreciation goes to my husband, Li Chen and my daughter, Joanna Chen for their understanding, inspiration, and loving support which helped me to achieve this degree.

Dedicated
to

My parents, Xihua Gao & Huixiang Zhang
My husband, Li Chen
&
My daughter, Joanna Chen

LIST OF TABLES

LIST OF FIGURES

FIGURE **PAGE**

ABBREVIATIONS

MMPs	matrix metalloproteinases
$ONOO^-$	peroxynitrite
TIMPs	tissue inhibitor of MMPs
IL-1β	interleukin-1β
TNF-α	tumour necrosis factor-α
IFN-γ	interferon-γ
NOS	nitric oxide synthase
iNOS	inducible nitric oxide synthase
BSA	bovine serum albumin
SDS	sodium dodecyl sulphate
EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glutamine tetra acetic acid
DTT	dithiothreitol
IgG	immunoglobulin G
Ab	antibody
DMSO	dimethyl sulfoxide
SOD	superoxide dismutase

CHAPTER I

INTRODUCTION

Systemic inflammatory response syndrome and advanced heart failure syndrome resulting from diverse pathologic insults involve severe deterioration of cardiac mechanical function. Pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) are implicated in the evolution of myocardial dysfunction under these pathological conditions, both in vitro and in vivo, in many different studies[28,106]. Those pro-inflammatory cytokines may be produced by macrophages, endothelial cells and cardiac myocytes. However, the biochemical mechanism of pro-inflammatory cytokine-induced myocardial contractile failure is not entirely clear. Understanding this is critically important for the development of pharmacological tools to prevent or treat immune-related cardiac diseases such as idiopathic dilated cardiomyopathies, infectious heart diseases, septic shock, and transplant rejection. The role of nitric oxide (NO), peroxynitrite ($ONOO^-$), and matrix metalloproteinases (MMPs) and their possible interactions in cytokine-mediated heart injury will be the subject of this introduction.

A. Nitric oxide

1. Physiological role of NO in heart

NO, a labile gas with a half-life of a few seconds in biological milieu, is generated from the five electron oxidation of L-arginine catalyzed by the NO synthase (NOS) enzymes[58]. There are at least three isoforms of NOS which have been named on the basis of the cell types they were originally discovered in: nNOS from neurons, eNOS from endothelial cells and inducible NOS (iNOS), an

isoform first characterized in macrophages and many other cell types in their response to immunological stimulation[66].

NO has fundamental physiological and pathophysiological roles in the cardiovascular system. Under normal circumstances NO is produced in cardiac myocytes, endocardial cells, and endothelial cells by eNOS[98]. This catalytic activity is tightly regulated by intracellular calcium levels and, as a result, only pico- to nanomolar concentrations of NO are produced for short periods of time. The physiological production of small quantities of NO in the heart acts to maintain coronary vasodilator tone[31], inhibit platelet aggregation and the adhesion of neutrophils and platelets to vascular endothelium[79]. NO also modulates cardiac muscle contractile function by its negative inotropic and chronotropic actions[39,95]. In addition, NO is a reversible inhibitor of mitochondrial oxygen consumption due to its effect on complex II of the respiratory chain[117].

The physiological role of NO in the heart is protective and multifaceted. NO stimulates soluble guanylate cyclase which reduces the intracellular concentration of Ca^{2+} partly through the activation of cyclic GMP-dependent kinase[102]. Increased levels of cGMP trigger a reduction of calcium concentration by enhancing extrusion of calcium and its sequestration into intracellular stores. The lowered intracellular Ca^{2+} concentration results in vasodilation and this vasodilation effect may be beneficial in ischemic tissue. NO can terminate the chain propagation reactions of lipid peroxidation during oxidative stress[85]. The NO-mediated inhibition of platelet and leukocyte activation and their subsequent

adhesion to the endothelial is important for the preservation of coronary perfusion and for reduction and /or prevention the inflammatory response.

2. Pathophysiological role of NO in the heart

In marked contrast to eNOS, iNOS is Ca^{2+}-independent and is expressed upon activation of cells with bacterial endotoxin and /or pro-inflammatory cytokines which activate the iNOS gene, causing de novo synthesis of iNOS protein[72,93,106]. This results in sustained (for several hours) and much higher rates of NO production (micromolar) in some cells, especially macrophages. The appropriate combination of pro-inflammatory cytokines stimulates iNOS expression in nearly any cell type thus studied, including the majority of cells of the heart: cardiac myocytes[93], endocardial endothelium[98], coronary endothelial cells[107] and vascular smooth muscle cells[101].

A growing body of evidence supports a role of nitric oxide in sepsis syndrome (a systemic response to infection with septic shock and multiple organ failure as its most severe manifestation) in clinical practice. Significant elevated plasma levels of stable NO metabolites (NO_2^- and NO_3^-) in septic patients, especially those with hypotension, are evidence of enhanced NO production[16]. It has been showed that iNOS is expressed in a variety of organs during septicemia and septic shock. Cardiac iNOS expression has now been shown to occur in immune-related cardiac disorders such as cardiac allograft rejection[118], myocardial infarction[112], viral myocarditis[50], cardiacpulmonary bypass[53], and it has also been detected in human heart biopsies from some heart failure

patients[13]. In these pathologies, NO is considered as a double-edged sword exerting both beneficial and detrimental effects. On one hand, NO is cytoprotective through vasodilator, platelet- and leukocyte- inhibitory effects. On the other hand, excessive NO may be detrimental through negative inotropic and chronotropic effects or through its subsequent conversion to $ONOO^-$. The detrimental effects of excessive amounts of NO in sepsis can be partially blocked by inhibiting NOS with L-NMMA[76]. Anti-hypotensive effects with reduced requirement for vasopressor agonists have been shown in the clinical treatment of septic shock with NOS inhibitors[76]. However, the management of NO overproduction in inflammatory heart disease has not translated into a viable therapeutic modality. The primary reasons for this failure were the use of non-selective NOS inhibitors and the difficulties in appropriately titrating these drugs[28]. Many patients received excessive doses of these inhibitors and, as a result, lost both iNOS and eNOS derived NO, thereby losing also the beneficial, physiological functions of NO.

B. Peroxynitrite in the heart

NO is necessary in small quantities for a variety of physiological functions, but potentially toxic in higher concentrations. However, it is now understood that many of the toxic actions of NO are not directly due to NO but are mediated through $ONOO^-$, the reaction product of superoxide anion and NO. In 1990, Beckman et al demonstrated that NO reacts with superoxide at a diffusion-limited rate to form $ONOO^-$ ($k = 6.7 \times 10^9\ M^{-1}s^{-1}$)[4]. The rate constant of formation of

$ONOO^-$ is more than three-fold faster than the dismutation of superoxide by superoxide dismutase ($k = 2 \times 10^9 M^{-1}s^{-1}$). Under physiological conditions, a very small amount of $ONOO^-$ is formed as the concentration of superoxide dismutase is in excess of NO (1 μM vs. 10 nM). However, under a variety of inflammatory conditions, where NO production is upregulated, either by acute increase in Ca^{2+}-dependent eNOS activity via changes in shear stress or high $[Ca^{2+}]_i$ which occurs during acute reperfusion and/or ischemia, or via cytokine-induced de novo expression of iNOS. Under such conditions, one can predict the formation of $ONOO^-$ and which will predominate over the dismutation of superoxide.

Activated macrophages[35], neutrophils[8], neuronal cells[44], vascular endothelial cells[43] and cardiac myocytes[48] have the capacity to synthesize $ONOO^-$. Many studies show that enhanced formation of $ONOO^-$ in the myocardium is cytotoxic to the heart and contributes to ischemia/reperfusion injury both in vitro[110,119] and in vivo[48]. Also, $ONOO^-$ was shown to disturb coronary circulation. 0.3–1000 μM $ONOO^-$ caused a concentration-dependent vasodilatation of the coronary vasculature in isolated rat hearts[109]. Nitrotyrosine was found in hearts from rats with autoimmune myocarditis[37]and in dogs with IL-1β-induced myocardial failure[71]. Extensive protein tyrosine nitration was also found in myocardial inflammation in humans[42] and can cause cardiomyocyte apoptosis[1,36]. Many of these studies show a correlation between $ONOO^-$ formation and deterioration of cardiac function. Furthermore, $ONOO^-$ has also been implicated in the pathogenesis of atherosclerosis[5], septic shock[42] and neurodegenerative diseases[5].

Peroxynitrite is a precursor to highly reactive oxidant species. Peroxynitrite is stable at alkaline pH, but at pH<8 it is protonated to form an unstable intermediate, peroxynitrous acid[5]. Peroxynitrous acid is highly unstable and its degradation by either heterolytic or homolytic cleavage to form highly reactive oxidants, including hydroxyl radical, nitrogen dioxide radical and nitronium ion[77]. Whether $ONOO^-$ causes damage within the local environment depends upon its concentration, site of formation, duration of exposure, and concentration of antioxidants at the site[60] such as plasma, serum albumin, or glutathione. Peroxynitrite causes its deleterious effect by acting on lipids[85], proteins[59], carbohydrates[61]and deoxyribonucleic acid[86] leading to structural damage, alteration of protein and enzyme function or cell death. In particular, $ONOO^-$ had been shown to inactivate some enzymes like aconitase[9], Na^+-K^+-ATPase[30], and creatine kinase[55]. In contrast, $ONOO^-$ is known to be a potent activator of some matrix metalloproteinases (MMPs) including pro-MMP-8[69] and pro-MMP-2[80].

Mediators of immune-related heart disease downstream of NO need to be uncovered and exploited as therapeutic targets. Recent studies have shown that reactive oxygen species (NO, $ONOO^-$) are implicated in the regulation of MMPs, therefore, I will now focus on MMPs and their potential contributions to inflammatory cardiac dysfunction.

C. Matrix metalloproteinases

1. General introduction

In 1962, Gross and Lapiere reported the first collagenolytic activity in amphibian tissue[29]. After nearly 40 years, more than 25 matrix metalloproteinases (MMPs) have been identified and characterized[33]. MMPs, also called matrixins, are a family of zinc-dependent endopeptidases. They are involved in the remodeling of the extracellular matrix of tissue during various physiological conditions such as embryogenesis, cartilage and bone repair, wound healing, and angiogenesis[115]. Enhanced activities of MMPs are implicated in a variety of pathological states including tumor invasion and metastasis, cardiovascular diseases, inflammatory diseases including arthritis, liver fibrosis, renal and lung disease, brain injury, stroke and connective tissue diseases[115]. The general classification of these MMPs is based on substrate specificity in vitro, leading to five distinct groups (Table 1.1). We still know very little about their exact in vivo substrate preferences. MMPs are synthesized and secreted in a latent proenzyme form (zymogen) and their activities are strictly regulated at three levels: transcription, activation, and inhibition/deactivation[108].

2. Matrix metalloproteinase-2

The concept which currently resides over MMP biology is that these enzymes act over prolonged periods of time to facilitate only changes in the extracellular matrix. More recently, however, there is increasing evidence that MMPs can also rapidly regulate diverse cellular functions which are independent of their effects on the extracellular matrix[115]. Among the matrix metalloproteinases, MMP-2 (pro-enzyme: proMMP-2 or progelatinase A, 72 kDa; active enzyme: MMP-2 or gelatinase A, 64 kDa) is unique in that it is normally

found in many cells, and has a cell surface mode of activation that differs from other other members of the family. Also, MMP-2 has been shown to rapidly regulate a variety of cellular functions on a second to minutes timescale. It is released within seconds from platelets during aggregation[90]. Inhibition of platelet MMP-2 activity by o-phenanthroline abolished non-ADP and non-thromboxane A_2 mediated pathway of aggregation. Fernandez-Patron et al. demonstrated that control of endogenous vascular contractile tone is mediated by the proteolytic effects of MMP-2 on big endothelin-1, forming the proteolytic product "medium" endothelin-1 and which is tenfold more potent a vasoconstrictor than big endothelin[19]. They also showed that vascular MMP-2 cleaves endogeous calcitonin gene-related peptide and promotes vasoconstriction, suggesting that it acts as a modulator of vascular function[20]. Furthermore, inflammatory signals are attenuated by the proteolytic action of MMP-2 on monocyte chemoattractant protein-3[54]. These novel interactions can be attributed to the fact that MMPs are proteinases which can potentially cleave some proteins. For example, MMP-2 cleaves a number of peptide bonds in denatured collagen such as Gly-Leu, Gly-Ile, Gly-Asn, Gly-Ser, etc.

The acute ability of MMP-2 to act on these substrates leads to the possibility that MMP-2 may also play an acute role in some diseased states, such as inflammatory heart disease. No information is available regarding the expression and release of MMP-2 from the heart following a pro-inflammatory insult. Therefore, this study was designed to investigate whether there is an

Members of the Matrix Metalloproteinases Family

Group name	MMP number	M_r latent/active (Da)
Collagenase		
Collagenase 1	MMP-1	52,000 / 42,000
Collagenase 2	MMP-8	85,000 / 64,000
Collagenase 3	MMP-13	52,000 / 42,000
Collagenase 4	MMP-18	53,000 / 42,000
Gelatinase		
Gelatinase A	MMP-2	72,000 / 64,000
Gelatinase B	MMP-9	92,000 / 84,000
Stromelysin		
Stromelysin 1	MMP-3	57,000 / 45,000
Stromelysin 2	MMP-10	54,000 / 44,000
Stromelysin 3	MMP-11	64,000 / 46,000
Membrane-type		
MT1-MMP	MMP-14	66,000 / 54,000
MT2-MMP	MMP-15	72,000 / 60,000
MT3-MMP	MMP-16	64,000 / 53,000
MT4-MMP	MMP-17	57,000 / 53,000
Other types		
Matrilysin	MMP-7	28,000/19,000
Metalloelastase	MMP-14	54,000/22,000

Table 1.1 Members of the matrix metalloproteinase family. There are more than 25 matrix metalloproteinases which have been identified and characterized. According to their structures and substrate preference in vitro, they can be subdivided into five distinct groups. This table indicates the most commonly used names and the molecular weights of the proenzyme and the active enzyme.

acute role of MMPs in the heart in the setting of pro-inflammatory cytokine-induced myocardial dysfunction. Under these circumstances, it may allow us to understand potential interactions with NO, $ONOO^-$ and MMPs.

3. Regulation of MMP-2 activity

a. Protein and gene structure

MMP-2 and MMP-9 have a similar tertiary protein structure[113]. Starting from the N-terminus, the following features are seen: 1) a signal peptide which allows secretion into the endoplasmic reticulum and eventual export from the cell, 2) a propeptide domain which maintains the enzyme in an inactive state (the cysteine residue in this sequence interacts with the zinc active site in the catalytic domain, thus keeping the proenzyme in its latent form), 3) a catalytic domain with a highly conserved HEXXHXXGXXH sequence which binds Zn^{2+} and Ca^{2+}, 4) fibronectin repeats which allow interaction with gelatin substrates (lacking in other MMPs), and 4) all MMPs except MMP-7 contain a hemopexin/vitronectin-like carboxyl terminal domain which facilitates the interaction between MMPs and tissue inhibitors of MMPs (TIMPs).

Recently, MMP-2 has been cloned[22]. Intensive studies in this field have shown that each MMP is tissue-specifically regulated at the gene level. In the MMP-2 gene, the striking difference from other MMP gene family members is that there is no TATA box or 12-O-tetradecanoylphorbol-13-acetate responsive element for the binding of transcription factor SP-1 in the promoter region. A potential binding site (CCCCAGGC) for the transcription factor AP-2 is located in

the first exon[34]. The TATA box plays a key role in the transcriptional regulation of most promoters. MMP-2 gene is considered as a "housekeeping" gene and it is widely expressed by many cells.

b. Tissue inhibitors of metalloproteinase

Tissue inhibitors of metalloproteinase (TIMPs) are endogenous inhibitors of MMPs. To date, four distinct TIMPs have been cloned and sequenced[26]. Table 2 shows some of their molecular and functional characteristics. Overall, TIMPs share 37-51% homology. The distinctive structure similarity is that they all possess 12 cysteine residues in conserved regions forming six disulfide bonds, which best resemble six "disulfide knots" in the tertiary structure and which play a major role in the inhibition of MMP activity. TIMPs form an important endogenous system for regulating actual MMP activity in vivo. In light of the potential importance of these inhibitory proteins to modulate MMP activity, the TIMP family of secreted proteins is a field of active interest in cardiovascular biology. Different TIMPs display a different pattern of expression and TIMP expression is diversely regulated. TIMP-1 is more easily inducible like its MMP counterpart MMP-9, while TIMP-2 is predominantly constitutive[26]. TIMP-3 is the only member of the TIMP family which is found exclusively in the extracellular matrix[111]. It is regulated in a cell cycle-dependent fashion in certain cell types and may serve as a marker for terminal differentiation[111]. There is disagreement in the expression sites of TIMP-4. It is reported to be highly expressed in human

Members of the tissue inhibitors of matrix metalloproteinase family

Name	Protein (kDa)	Major functions
TIMP-1	28.5	Inhibit MMPs Bind pro-MMP-9 Anti-angiogenesis Inhibit apoptosis
TIMP-2	21.0	Inhibit MMP-2 Bind MT1-MMP and pro-MMP-2 (activate pro-MMP-2) Anti-angiogenesis Inhibit apoptosis
TIMP-3	21.0	Inhibit MMPs Matrix-associated Bind MT1-MMP Induce apoptosis Anti-angiogenesis
TIMP-4	22.0	Inhibit MMPs Bind pro-MMP-2 highly expressed in the heart and brain

Table 1.2 Tissue inhibitors of matrix metalloproteinase. Four TIMPs have been cloned and purified. Each TIMP is encoded by a unique gene, yet all share both structural and functional similarities. The TIMPs are low-molecular-weight proteins (20-30 kDa) which have multiple biological effects such as regulating MMP activity, cell proliferation, apoptosis, and anti-angiogenesis.

adult heart and undetectable in other organs whereas the murine TIMP-4 was shown to be expressed in a much broader range of tissue[116].

Although TIMPs are considered to be endogenous inhibitors of MMPs, the in vivo function of these proteins may not be as simple as their names. First, TIMPs participate in the process of MMP activation (see 3.3). A second role of TIMPs that is independent of modulating MMP activational states is through effects on cell growth. For example, TIMP-1 and TIMP-2 have been shown to stimulate a growth response in fibroblast cell cultures in a concentration-dependent manner[40]. Thus, it is possible that changes in TIMP levels within the myocardium may have multiple biological effects that would be relevant to the cardiomyopathic disease process.

c. MMP activation

All MMPs are secreted in a latent form. The activation process is important in the regulation of activity of MMPs, especially for MMP-2, which is not so tightly regulated at the transcriptional level compared to MMP-9. The conversion from proenzyme to active form is believed to be a two-step process either in vitro or in vivo. Accordingly, the first step is disruption of a cysteine-zinc bond that can be achieved either by proteolytic or nonproteolytic ways. By definition, it is by cleavage of the propeptide, or by conformational changes induced by oxidant stress molecules such as $ONOO^-$. This procedure was first modeled by Van Wart and Birkedal-Henson as a "cysteine switch" hypothesis[108]. In the second step,

the intermediate active enzyme trims off additional residues autocatalytically resulting in the active enzyme with 8-10 kDa lower molecular mass (Figure 1).

MMP-2 has a unique activation pathway. The proMMP-2 is secreted as a 72 kDa latent form bound with TIMP-2. In 1990, Brown et al. presented the first clue to elucidate the cellular activation mechanism of MMP-2[6]. They found that proMMP-2 was retained at the cell surface, a feature which was likely to have important implications in vivo since it would allow migrating/invading cells to restrict the activation of the locally produced proenzyme/TIMP-2 complex to the migration or invasion area boundary[7]. Anchoring of the proMMP-2/TIMP-2 complex and activation was achieved by an activator localized to the cell surface. In 1994, Sato et al. cloned and named this activator membrane type matrix metalloproteinase (MT-MMP)[89]. Very recently, a radioactive binding study suggested that the process started from TIMP-2 binding to the active site of MT1-MMP after which proMMP-2 was recruited to the complex through the interaction of the carboxy-terminal domains of TIMP-2 and proMMP-2[121]. Accordingly, the trimolecular complex was able to trigger the autocleavage of the proenzyme[121]. Other components have also been suggested to be involved in the cell surface interaction of proMMP-2, such as integrin $\alpha_v\beta_3$ which can function as an extracellular matrix receptor to provide powerful "outside-in" cellular signals to regulate cellular function[83].

d. NO and $ONOO^-$ in the regulation of MMPs

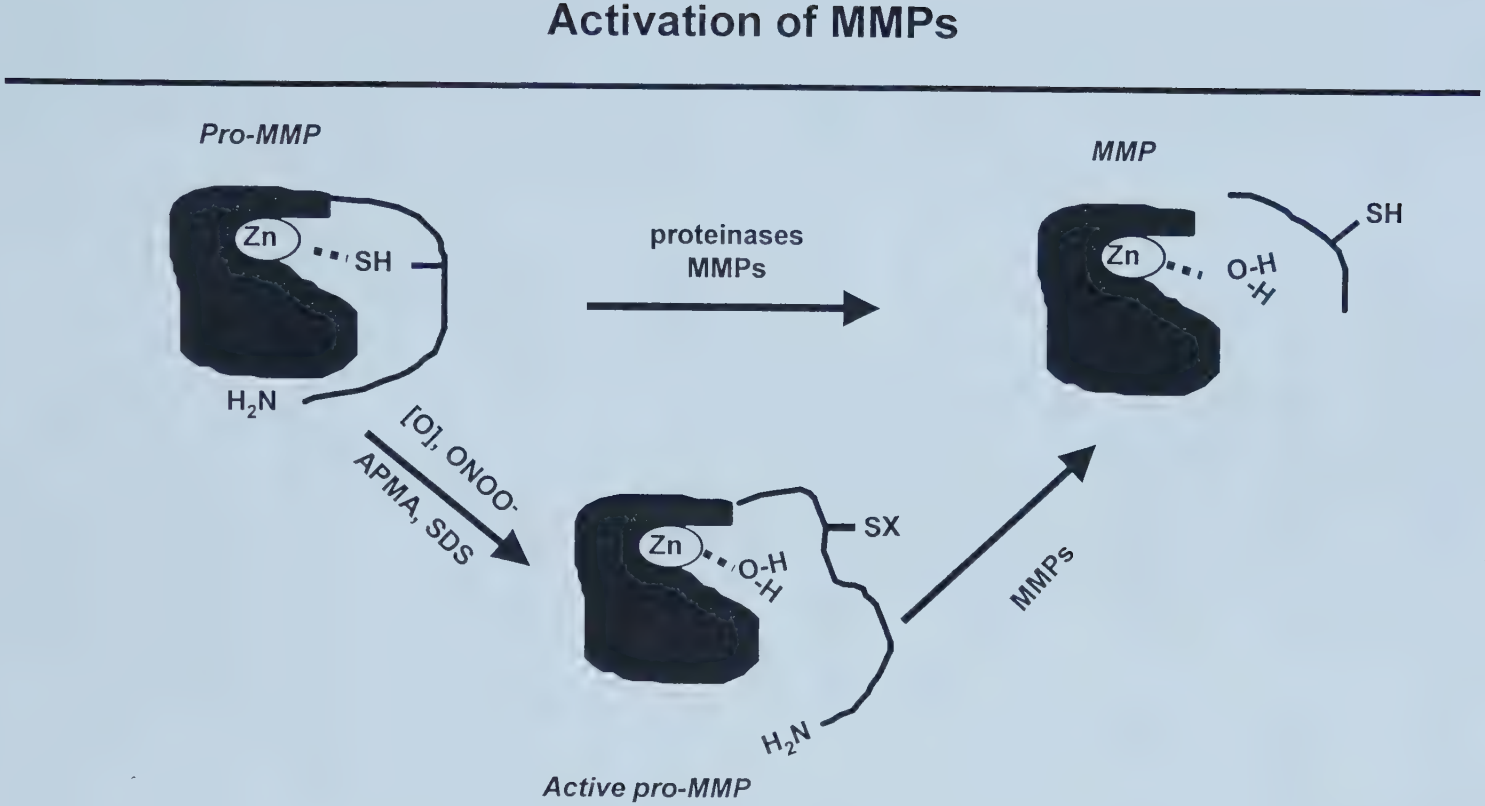

Figure 1.1 Activation of MMPs. MMPs are synthesized as pro-enzymes. The activation of the proenzyme can be achieved either through the breakage of a cysteine-Zn bond through proteolytic cleavage of part of the propeptide or by conformational changes induced by oxidant stress molecules such as superoxide and $ONOO^-$. (Modified from Parks WC, Mecham RP: Matrix Metalloproteinases, San Diego: Academic Press;1998; pp.87)

Transcriptional regulation of MMPs: The activation of AP-1, a heterodimeric complex of fos and jun protein, is required for the synthesis of MMPs. In 1995, Lo and Cruz reported that reactive oxygen species were involved in the induction of c-fos expression by TNF-α and basic fibroblast growth factor (bFGF) in the cultured bovine articular chondrocytes[51]. The authors demonstrated that both TNF-α and bFGF were capable of stimulating reactive oxygen species production by chondrocytes. The antioxidants N-acetylcysteine and ascorbic acid attenuated the induction of c-fos expression, whereas no effect was seen with a NOS inhibitor or NO donor. They did not show direct evidence between changes of c-fos and changes of MMPs. Sasaki et al have been the sole group to report NO mediation of MMP gene expression[88]. Rabbit articular chondrocytes were cultured and treated with IL-1, a pro-inflammatory cytokine. This induced the expression of iNOS and MMP-9 mRNA and protein. Interestingly, the administration of L-NMMA (a nonselective NOS inhibitor) inhibited expression of MMP-9 protein and mRNA. These results suggest that cytokine-stimulated NO production can directly upregulate MMP gene expression.

One potential shortcoming of the reports was the attribution of these effects to NO when they were more likely to be mediated by $ONOO^-$. For instance, cells in the previous paper were treated with pro-inflammatory cytokines and iNOS expression was induced; thus, based on the kinetic arguments above, it is conceivable that large amounts of $ONOO^-$ were produced

in this system. Clearly, further investigation will identify which molecule (NO or $ONOO^-$) mediates MMP transcription and by which mechanisms they act.

Post-transcriptional activation of MMPs: Early reports on the role of NO in the activation of MMPs were conflicting. Observations on cytokine and endotoxin stimulated human and bovine chondrocytes and rat mesangial cells showed induction of iNOS expression and activity as well as increased stromelysin, collagenase, and MMP-2 activities[64,103]. Addition of L-NAME to IL-1β or endotoxin stimulated cells decreased MMP-2 activity. An in vivo study showed that a ten week course of orally administered N-iminoethyl-L-lysine, a selective inhibitor of inducible NOS, significantly decreased MMP activities in a dog osteoarthritis model[74]. In contrast, Sawicki et al. reported that NO inhibits the release of MMP-2 from human platelets. Using the NO donor S-nitroso-N-acetylpenicillamine (SNAP,0.01-10 μM), they demonstrated a concentration-dependent reduction of the release of MMP-2 from stimulated platelets[90]. Radomski et al. reported that Ca^{2+}-independent NO activity and MMP-2, -9 activity were increased in hyperoxia-induced lung damage in newborn rats[78]. The L-NAME treatment reduced lung edema and epithelial proliferation, but enhanced the activities of MMPs[78]. However, as with the previous mentioned chondrocytes and mesangial cell study[64,103], the discrepancy may be explained by $ONOO^-$ formation. Cytokine and endotoxin stimulation results in NO and superoxide production which favors $ONOO^-$ formation and may have been responsible for MMP activation. So the investigators should not exclude the possible action of superoxide and $ONOO^-$ in

the above studies[64,103]. This contention is supported by later reports, which demonstrated that NO has little to no ability to activate purified MMPs (that is, in cell free conditions where $O_2^{\cdot-}$ is not present).

Recent observations have more definitively shown that $ONOO^-$ activates MMPs. Okamato et al. demonstrated that purified MMP-8 can be activated in a concentration-dependent manner when exposed to synthetic $ONOO^-$[69]. This effect was found to be extremely sensitive as three brief exposures of MMP-8 to 1 μM $ONOO^-$ resulted in its activation. As well, continuous infusion of 20 μM $ONOO^-$ produced a time-dependent increase in MMP-8 collagenolytic activity. Moreover, $ONOO^-$ is far more potent than NO in activating proMMP activity. As little as 1-20 μM $ONOO^-$ cause strong activation of purified proMMP whereas 10 mM NO was necessary for even modest activation. In addition, $ONOO^-$ activated proMMP without a change in molecular weight. Similar results were seen in another study, co-incubation of human vascular proMMP-2 with $ONOO^-$ significantly increased the activation of MMP-2[80].

Only one report has shown a decrease of MMP activity after exposure to $ONOO^-$[70]. However, these effects may be attributed to the high concentrations of $ONOO^-$ (mM) used in this study. At these extreme concentrations, $ONOO^-$ may compromised the integrity of the MMP protein and its catalytic activity through nitration, oxidation, or even direct proteolytic actions.

$ONOO^-$ activation of MMPs is likely to be mediated through the disturbance of the "cysteine switch" hypothesized by Birkedal-Hansen[108]. The Zn^{2+} atom at the heart of all MMP active sites complexes with a cysteine residue

at the highly conserved region of the propeptide domain. Disruption of this Cys-Zn^{2+} bond allows proteolytical activation. $ONOO^-$ could disrupt this bond by oxidation of the cysteine moiety or nitration of proximal tyrosine residues. Rajagopalan et al. demonstrated that nitration of MMPs by $ONOO^-$ can occur in vitro[80]. Incubating MMP-2 with $ONOO^-$ increased activity and the appearance of nitrotyrosine residues within the enzyme in a concentration-dependent manner.

TIMPs: A final and important control point of MMP activity is the inhibition of activated enzyme. The TIMPs are low molecular weight proteins (20 to 30 kDa) and four members have been characterized so far[26]. The N-terminal domain of TIMPs binds to the highly conserved catalytic region of MMPs to form a non-covalent bond which inhibits their activity[120]. $ONOO^-$ may alter the structural and binding characteristics of TIMPs, resulting in their dissociation from proMMPs, and hence their activation. In vitro, it has been shown that $ONOO^-$ concentration-dependently inactivates the inhibitory activity of TIMP-1 towards MMP-2 (IC_{50}=50 μM)[23]. Aside from the TIMPs, $ONOO^-$ has been shown to inhibit α_1-protease inhibitor, a potent inhibitor of MMP-8[52,59]. As a consequence of $ONOO^-$ inactivating MMP inhibitors and activating MMPs, the balance of MMPs and TIMPs would be shifted, resulting in an increase in net proteolytic activity during pathological states.

4. Pathophysiological implications of MMPs in the heart

Activation of MMPs is implicated in cardiac injury resulting from diverse pathologic insults such as ischemia, infarction, and inflammation. Cheung et al[10] found that MMP-2 was rapidly released into the perfusate during the first minute of reperfusion after ischemia by using isolated rat hearts. Inhibitors of MMP or anti-MMP-2 antibody improved the recovery of post-ischemic mechanical function[10]. Li et al[46]demonstrated a selective downregulation of both TIMP-1, and -3 transcripts and proteins along with upregulation of myocardial MMP-9 gelatinolytic activity in cardiomyopathic ventricles. The same group also showed TIMPs were different regulated in cardiac myocytes subjected to proinflammatory cytokines TNF-α and IL-1β along with upregulation of gelatinolytic activity[47]. A number of evidence supports that MMPs directly mediate crucial steps during the pathogenesis of myocardial infarction and may determine the clinical outcome. MMP activity rapidly increases in the myocardium during infarction and remains elevated through the healing phase[105]. Also infarcted hearts from MMP-9 deficient mice show a reduction in both early myocardial rupture[32] as well as progressive ventricular dilation[14]. Experiments from several laboratories indicate that in both humans and experimental animal models of heart failure that there is increased activation of myocardial MMPs and that inhibition of MMPs can block ventricular dilation[75,81,100]. Furthermore, Kim et al[41] demonstrated that transgenic overexpression of the human MMP-1 gene in the mouse ventricle leads to myocyte hypertrophy and ventricular dysfunction. Based on the results of this and several recent studies, it will be important to identify both upstream mechanisms for myocardial MMP activation and their downstream targets.

C. Conclusions

Pro-inflammatory cytokines enhance the myocardial generation of $ONOO^-$ through concerted increase in both NO and superoxide, which can cause the depression in contractile function, yet the mechanism by which this occurs is unknown. Upregulation of MMP activity and downregulation of TIMP activity and/or expression may contribute to the pathological changes in cardiac injury resulting from diverse pathological insults. Pharmacological inhibition of MMP activity or scavenging peroxynitrite might then be able to improve the recovery of myocardial dysfunction subjected to diverse pathological insults such as pro-inflammatory cytokines.

D. Thesis hypothesis and objectives

Thesis hypothesis:

- MMPs activity increases in hearts during pro-inflammatory cytokine-mediated injury as a result of $ONOO^-$ formation

Thesis objectives:

- To determine the effect of pro-inflammatory cytokines on myocardial function in the isolated heart

- To determine NOS activity and $ONOO^-$ formation in pro-inflammatory cytokine-induced myocardial dysfunction

- To determine changes in MMP activity/secretion in both perfusate and myocardial tissue in pro-inflammatory cytokine-induced myocardial dysfunction

- To determine the effects of a $ONOO^-$ scavenger or MMP inhibitors on improving myocardial function during cytokine-induced myocardial dysfunction

- To determine changes in TIMP-4 protein expression in cytokine-induced myocardial dysfunction

CHAPTER II

MATERIALS AND METHODS

A. Materials

Polyclonal anti-peptide antibodies were generated in rabbits using synthetic peptide corresponding to a fragment (N-G-K-E-Y-N-S-[ABA]-T) of the gelatin-binding domain of human MMP-2[91] (a gift from Dr Mieczyslaw Wozniak, Department of Clinical Chemistry, Medical University, Wroclaw, Poland). Rat IgG (Sigma) was used as a negative control for the antibody against MMP-2.

Conditioned culture medium from a human fibrosarcoma cell line HT-1080 (American Type Culture Collection, Rockville, MD), which contains large amounts of MMP-2, was used as a standard. HT 1080 cells were maintained at 37°C in a humidified chamber containing 5% CO_2 in air in Eagle's minimum essential medium with 10% fetal calf serum at a concentration of 5×10^5 cells/ml. The cells were washed 3 times with serum-free media and incubated at 37°C for 24 hr with the phorbol ester 12-o-tetradecanoyl-phorbol-13-acetate (0.1 μM). The cell-conditioned medium was separated from cells by centrifugation (1500 g for 10 min at room temperature), and the supernatant was aliquoted and stored at -80 °C.

The MMP inhibitors PD166793 (Parke-Davis Pharmaceutical Research, Ann Arbor, MI) and Ro31-9790 (Roche Discovery Welwyn, Welwyn Garden City, Hertfordshire, UK) and affinity-chromatography purified rat myocardial TIMP-4[104] (0.2 mg/ml stock concentration in 30 mM Tris-CL, 0.1% SDS, 0.1 M glycine, Dr. Suresh Tyagi, University of Mississippi Medical Center, Jackson, MS) were kind gifts from the sources indicated. The following reagents were purchased as follows: recombinant human IL-β, TNF-α and IFN-γ (R & D Inc., Minneapolis,

MN); insulin (Eli Lilly Canada Inc, Toronto, ON); bovine serum albumin (Boehringer Mannheim, Indianapolis, IN); rabbit anti human TIMP-4 antibody (Chemicon, Temecula, CA); FeTPPs (5,10,15,20-4–sulfonatophenyl-porphyrinato-iron III, Calbiochem, San Diego, CA) and its inactive form TPPs (Porphyrin Products, Logan, UT); L-[U-^{14}C]-arginine monohydrochloride, 278 mCi mmol^{-1} (Amersham, Buckinghamshire, England); other reagents were purchased as indicated; the rest was obtained from Fisher Scientific or Sigma .

B. Animal model

Adult male Sprague-Dawley rats weighing 250-330 g were used throughout the studies. These animals were maintained in the Health Science Laboratory Animal Services of the University of Alberta. Animals were provided with normal rat lab chow and water. This investigation conforms with The Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (revised 1993) and approved by the Health Sciences Animal Policy and welfare Committee of University of Alberta.

C. Heart perfusions

1. Animal preparation Male Sprague-Dawley rats weighing were anesthetized with 60 mg/kg intraperitoneal injection of sodium pentobarbital (Euthanyl^{TM}, Bimedia MTC, Cambridge, ON). A transverse incision was made to open the abdominal cavity. The diaphragm was transected and the chest was opened by an incision cut along the sternum. The pericardium was cut and pulled

away from the heart. The heart was picked up and then excised with the lungs and quickly rinsed in ice-cold Krebs-Henseleit solution for a few seconds. The aorta was cannulated onto the perfusion rig and tied firmly with suture.

2. Langendorff Perfusion The retrograde perfusion of the heart was performed as first described by Langendorff[45]. Hearts were perfused at constant hydrostatic pressure at 70 mmHg with a modified Krebs-Henseleit solution containing (in mM): NaCl (118), KCl (4.7), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $NaHCO_3$ (25), glucose (11), $CaCl_2$ (1.75), EDTA (0.5). The perfusate was maintained at 37°C and was continuously gassed with 95% O_2-5% CO_2 to give a final pH of 7.4. This solution was used for the initial perfusion while the heart was set-up for perfusion in the working mode. The Langendorff perfusion allows for the washout of blood and traces of anesthetic as well as equilibration of the solution salt and substrate concentration with those of the interstitial fluid and is important to allow the heart to recover from the brief period of hypoxia that was associated with excising the heart.

3. Working Mode Perfusion The isolated working heart method was originally described by Neely et al[67]. After 5 min equilibration in Langendorff perfusion, the heart was rotated on the aortic cannula in order to position the atrial cannula close to the opening of the pulmonary vein into the left atrium. Any remnants of the lungs, connective tissue, or thymus were trimmed from the heart which also aided the exposure of the atrial opening for cannulation. Once the left

atrium was cannulated and firmly tied, the retrograde Langendorff perfusion was stopped and, simultaneously, switched to the working heart model by clamping the aortic inflow line from the Langendorff reservoir and opening the left atrial inflow and aortic outflow lines connected to the working mode perfusion reservoir. The pulmonary artery was carefully snipped to allow free outflow of the coronary circulation. The composition of the working mode perfusion solution was: 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, 5 mM pyruvate, 100 μU/ml insulin, 1.75 mM Ca^{2+}, 0.5 mM EDTA, 0.1% BSA, and 0.3 mM L-tyrosine. The latter substrate reacts with $ONOO^-$ to form the fluorescent product dityrosine. To avoid precipitation, L-tyrosine was dissolved in 2 ml of 1 N NaOH and added to working mode perfusion solution. The perfusate was delivered from the oxygenator (supplied with 95% O_2-5% CO_2) into the left atrium at a hydrostatic preload pressure equivalent to 9.5 mmHg. The hydrostatic afterload pressure was set at a column height equivalent to 70 mmHg. Hearts were either paced at 300 beats/min with a Grass SD9 stimulator (for the MMP inhibitors study, Chapter IIIA) or spontaneous beating (for $ONOO^-$ scavenger study, Chapter IIIB). The 110 ml recirculating perfusate was ejected from the hearts into a compliance chamber (containing 1 ml of air) and into the aortic outflow line. All the perfusate reservoirs were water-jacketed to keep the perfusate temperature constant at 37°C throughout the perfusion protocol. Heart rate and peak systolic pressure were measured with a TSD 104 Grass pressure transducer in the aortic outflow line and recorded in real time using the AcqKnowledge III data-acquisition system (Biopac System,

Goleta, CA). Cardiac output and aortic flow were measured using two ultrasonic flow probes (2N390, Transonic Systems, Ithaca, NY) in the preload and afterload lines, respectively. Coronary flow was calculated as the difference between cardiac output and aortic flow. Cardiac work (cardiac output X peak systolic pressure) was used as an index of mechanical function of the heart.

D. Experimental groups

After 20 minutes equilibration in the working mode (referred to as 0 min), hearts were perfused for 120 minutes in the absence (Control, n=7) or presence of 5 ng/ml recombinant human IL-1β, 9 ng/ml recombinant rat IFN-γ, and 20 ng/ml recombinant human TNF-α (Cytokine, n=10) added to the recirculating perfusate at t = 0 min. Perfusate samples were taken at -1, 1, 30, 60, 90, and 120 min of perfusion to determine MMP activity and dityrosine levels. At the end of the perfusion protocol (t = 2 hr), heart ventricles were rapidly frozen with Wollenberger clamps cooled to the temperature of liquid N_2, followed by brief immersion into liquid N_2 and stored at -80°C for later processing. Additional series of hearts were perfused with pacing (300 beats per minute) for 2 hr with MMP inhibitors (Ro31-9790, 3 μM or PD166793, 2 μM), DMSO vehicle (final concentration in perfusate was 0.3% v:v), neutralizing MMP-2 antibody (30 μg/ml), or rat IgG (30 μg/ml, Sigma), respectively, in the presence or absence of cytokines. Other spontaneously beating hearts in the presence or absence of cytokines were treated with $ONOO^-$ scavenger (FeTPPs, 50 μM) or its inactive

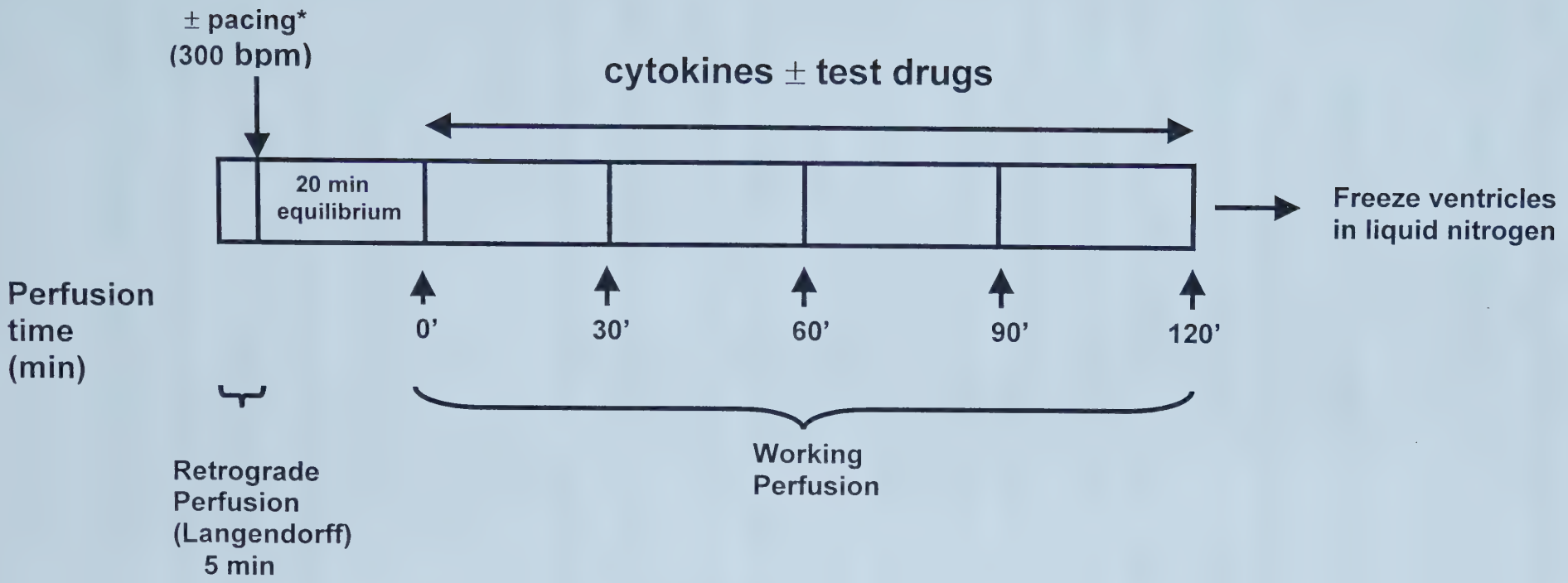

Figure 2.1 Isolated working rat heart perfusion protocols. Retrograde perfusion was initiated immediately after heart isolation for 5 minutes and was followed by 2 hr working mode perfusion. Cytokines, with or without drug treatment, were added at time 0. Effluent samples were drawn at time –1, 1, 30, 60, 90, and 120 min. * Study with FeTPPs or TPPs was done with spontaneously beating hearts.

form TPPs (50 μM). All reagents were added at t = 0 min. A summary of the perfusion protocol is given in Fig 2.1.

E. Measurement of cardiac peroxynitrite generation

We have previously determined that the measurement of dityrosine, a fluorescent product, formed by the reaction of peroxynitrite with tyrosine[17] in heart perfusate supplemented with 0.3 mM L-tyrosine, can be used as an estimate of peroxynitrite generation and that tyrosine at this concentration does not affect cardiac mechanical function. Samples of the perfusate were collected at –1, 1, 30, 60, 90, and 120 min of perfusion, placed on ice, and protected from light until analysis. The formation of dityrosine was analyzed by fluorescence spectroscopy (λ_{ex} = 320 nm and λ_{em} = 410 nm; excitation slit = 5 nm and emission slit = 10 nm) at room temperature using a Shimadzu RF 5000 spectrophotofluorometer. It has been showed in our previous study that solutions of authentic dityrosine standard (0.01-1.0 μM) in Krebs-Henseleit solution (pH 7.4) had a linear relationship between fluorescence intensity and concentration[119].

F. Preparation of heart extracts

For zymography and Western blot experiments, the frozen ventricular tissue was powdered with a pestle and mortar cooled to the temperature of liquid N_2. A portion of this tissue powder (150-200 mg) was placed in four volumes of ice-cold homogenization buffer (50 mM Tris-HCl, 3.1 mM sucrose, 1 mM

dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, and 0.1% Triton X-100) and then homogenized with an Ultra-Turrex dispenser using three strokes of 5 s duration each. The homogenate was centrifuged at 8,500 g at 4°C for 5 min and the supernatant was collected. For determination of NOS activity Triton X-100 was omitted from the homogenization buffer and the homogenate was centrifuged at 1000 g at 4°C for 5 min to obtain the crude supernatant fraction.

G. Protein Determination

Protein content of the heart extract was determined by the bicinchoninic acid method using bovine serum albumin as a standard (P-0914, Lot 20k 8801, Sigma). 10 μl of heart extract was diluted with 190 μl water (1:20) and was mixed with bichinchonic acid reagent (10 μl). The bichinchonic acid reagent was prepared freshly by mixing bichinchonic acid solution with $CuSO_4$ solution in a 50:1 ratio (v:v). Dilutions of bovine serum albumin (1.5 μg/μl –10 μg/μl) in water were prepared in order to determine a standard curve. All samples were pipetted into the wells of a 96 well microplate in triplicate and incubated at 37°C for 30 min. Immediately following incubation the optical density of all samples was read at room temperature at 560 nm using an UV-Max microplate reader (Molecular Devices Corporation, Menlo Park, CA). Protein concentrations were calculated from the bovine serum albumin standard curve.

H. Measurement of NOS activities

NOS activities in the heart extract were measured by the formation of radiolabelled L-[^{14}C] citrulline from L-[^{14}C] arginine, essentially as described by Knowles et al[93]. The heart extract (20 μl) was incubated in duplicate in cocktail assay buffer (100 μl; final concentration of reagents as diluted with heart extract are: 50 mM L-valine, 50 mM KH_2PO_4, 1.2 mM $MgCl_2$, 0.24 mM $CaCl_2$, 0.1μM NADPH, 1 mM L-citrulline, 18 μM L-arginine, 10 μM 5,6,7,8-tetrahydrobiopterin, 1.25 mM dithiothreitol, and 2 μM L-[U-^{14}C]-arginine) for 25 min at 37°C in the presence or absence of either EGTA (1 mM) or EGTA plus N^G-monomethyl-L-arginine (1 mM) to determine the level of both Ca^{2+}-dependent and Ca^{2+}-independent NOS activities. To prepare resin, 1 g of AG 50W-X8 resin (200-400 mesh, Biorad, Hercules, CA) for every 3 reaction tubes was weighed out and 60 ml of 2N NaOH is added in a glass beaker to activate the resin. This resin was agitated by swirling and then allowed to settle in order to decant the NaOH solution. The resin was then washed at least three times with water until pH < 7.0. After decanting the last wash, a measured amount of water (1 ml/sample) was added to the resin and was mixed. The pH was checked to ensure neutral or slightly acidic pH.

The assay steps are as followings:

- Number 2 ml microcentrifuge tubes. Samples are prepared in duplicates and each tissue sample requires a set of 3 duplicates (6 tubes).
- Add 100 μl of cocktail buffer directly into the bottom of each tube.
- Pipet 5 μl of EGTA stock solution into tube 3 and 4 of each set of six tubes.

- Add 5 μl of EGTA + L-NMMA stock solution into tubes 5 and 6 of each set of 6 tubes.
- Place tubes in 37°C water bath and incubate for 10 minutes.
- Add 20 μl of heart extract into each of six tubes, vortex briefly and incubate for 25 min at 37°C.
- Add 1 ml of activated resin suspension to tubes to stop the reaction (at neutral pH, resin quantitatively absorbs L-arginine which exists in a cationic form whereas L-citrulline is uncharged and remains in solution). Remove tubes and place at room temperature.
- Add 300 μl of water to each tube.
- Spin tubes at 10,000 g for 2 minutes at room temperature.
- Remove 700 μl of supernatant from each of the tubes, carefully avoiding the uptake of any of the resin beads, and place into small counting vials.
- Add 2 ml of scintillation liquid (ScintiSafe^{TM}30%, Fisher, Fair Lawn, NJ) to each vial and count as ^{14}C for 10 min.

The average dpm of the first duplicate (tubes 1 and 2) is used to determine the total NOS activity. The average dpm of the second duplicate (tubes 3 and 4) is subtracted from the average of the first duplicate and this difference is used for Ca^{2+}-dependent NOS activity. The difference between the average dpm of the second duplicate and that of the third duplicate (tubes 5 and 6) is used for Ca^{2+}-independent activity. NOS activities were expressed in picomoles per min per milligram of protein.

I. Measurement of MMPs by zymography

Gelatin zymography was performed as described[10]. Briefly, perfusate samples or myocardial extracts (20 μg) were mixed with non-reducing sample loading buffer, and applied to 7.5% polyacrylamide gel copolymerized with 2 mg/ml gelatin. 20 μg protein was loaded in each lane. After electrophoresis, gels were rinsed in 2.5% Triton X-100, 20 min washing for three times to remove SDS. Then the gels were washed twice in incubation buffer for 20 min each at room temperature. The composition of the incubation buffer was (in mM): Tris-HCL (50), $CaCl_2$ (5), NaCl (150) and 0.05% NaN_3. The gels were then kept in incubation buffer at 37°C for 18 or 48 hr for the perfusate and heart extract samples, respectively. After incubation, gels were stained in staining solution (2% Coomassie Brilliant blue G, 25% methanol, 10% acetic acid) for 2 hours and then destained for 1 hour in destaining solution (2% methanol, 4% acetic acid). Zymograms were scanned using an HP6100 scanner (Hewlett-Packard), and the band intensities were analyzed by Sigma gel software. MMP activities were expressed as a percentage of the 64 kDa band in the standard (HT 1080 cell conditioned medium).

J. Western blot analysis

Heart extract was diluted with 50 μL protein sample buffer (30% v/v glycerol, 2% wt/vol SDS, 0.13 M Tris, 0.1 mg/ml bromphenol blue, and 4% v/v 2-mercaptoethanol, pH=6.8) to give a final protein concentration of 0.1 μg/μl in a total volume of 300 μl. The samples were boiled for 5 min and then stored at

-20°C until assay. 1 μg protein was applied to 12% polyacrylamide gels and electrophoresed using a Mini-protean II electrophoresis cell (BioRad, Hercules, CA) at a constant voltage of 120 V. Electrophoresis was carried out under nonreducing conditions. After electrophoresis, the proteins were electroblotted to polyvinylidene membranes (Biorad) using the Semi-Dry Transfer Cell (Biorad) for 30 min at 25 V using transfer buffer (20% methanol, 0.125 M Tris-HCl, 0.96 M glycine, 0.05% SDS). The gel was stained for 30 min with Coomassie blue staining solution (0.05% wt/v Coomassie blue G-250, 20% v/v isopropanol, 10% v/v acetic acid) and destained with destaining solution (7.5% acetic acid, 10% methanol, v/v) for 2 hr to visualize the remaining untransferred proteins. This procedure was helpful to determine the efficiency of the transfer protocol. In order to reduce non-specific binding of the antibody, the membrane was incubated with 10% skim milk powder (wt/vol) in TTBS buffer (20% v/v Tween, 0.01 M Tris, 0.1 M NaCl) for 2 hr at room temperature. The blot was then incubated for 2 hr with rabbit anti human TIMP-4 antibody, diluted 1:4000 in blocking buffer. The blots were washed 4 times for 5 min in TTBS buffer before incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Transduction Laboratories, Mississauga, ON) diluted 1:2000 with blocking buffer. After that, the blots were washed four times for 5 min each in TTBS buffer. All of the membrane processing was done at room temperature with constant agitation. The antibody-bound protein was visualized using reagents from an enhanced chemiluminescence detection kit (ECL, Amersham, Buckinghamshire, England) and were autoradiographed using scientific imaging film (BioMax MR-1film,

Eastman Kodak, Rochester, NY). Rat myocardial TIMP-4 was used as a positive control. Band densities were analyzed by Sigma gel software and expressed as percentage of the TIMP-4 standard band density.

K. Statistical analysis

Results are expressed as means ± SEM for n hearts. As appropriate, Student's t-test, one-way or two-way (simple or repeated measures) ANOVA were used for statistical comparisons followed by Tukey's post hoc test. Differences were considered significant at $P < 0.05$.

CHAPTER III

RESULTS

A. Inhibition of MMP activity attenuates cytokine-induced myocardial dysfunction

1. Effect of pro-inflammatory cytokines on cardiac mechanical function

The combination of three pro-inflammatory cytokines (5 ng/ml IL-1β, 20 ng/ml TNF-α, 9 ng/ml IFN-γ) was investigated in isolated working rat hearts paced at 300 bpm. Figure 3.1 shows the time course of changes in cardiac function, measured as cardiac work (cardiac output X peak systolic pressure), in control and cytokine-treated hearts. Cardiac work in control hearts remained stable for the first 90 min of perfusion, followed by a gradual decline over the last 30 min, yet remained at 85% of cardiac work measured at 0 hr ($P>0.05$, one way ANOVA). Cytokine-treated hearts remained stable for the first 60 min of perfusion and declined significantly in cardiac work between 60 and 120 min of perfusion to 37% of the original cardiac work ($P<0.05$, one way ANOVA). The depression in cardiac work in cytokine-treated hearts became significant at 90 min and remained so for the remaining perfusion time ($P<0.05$, two way repeated measures of ANOVA). Coronary flow (Table 3.1) was significantly depressed in cytokine-treated hearts compared to 0 min and to the corresponding control hearts at t = 2 hr ($P<0.05$, one way ANOVA). These results show that this combination of pro-inflammatory cytokines at these concentrations causes a clear reduction in cardiac mechanical function compared to control hearts as described previously[18,72].

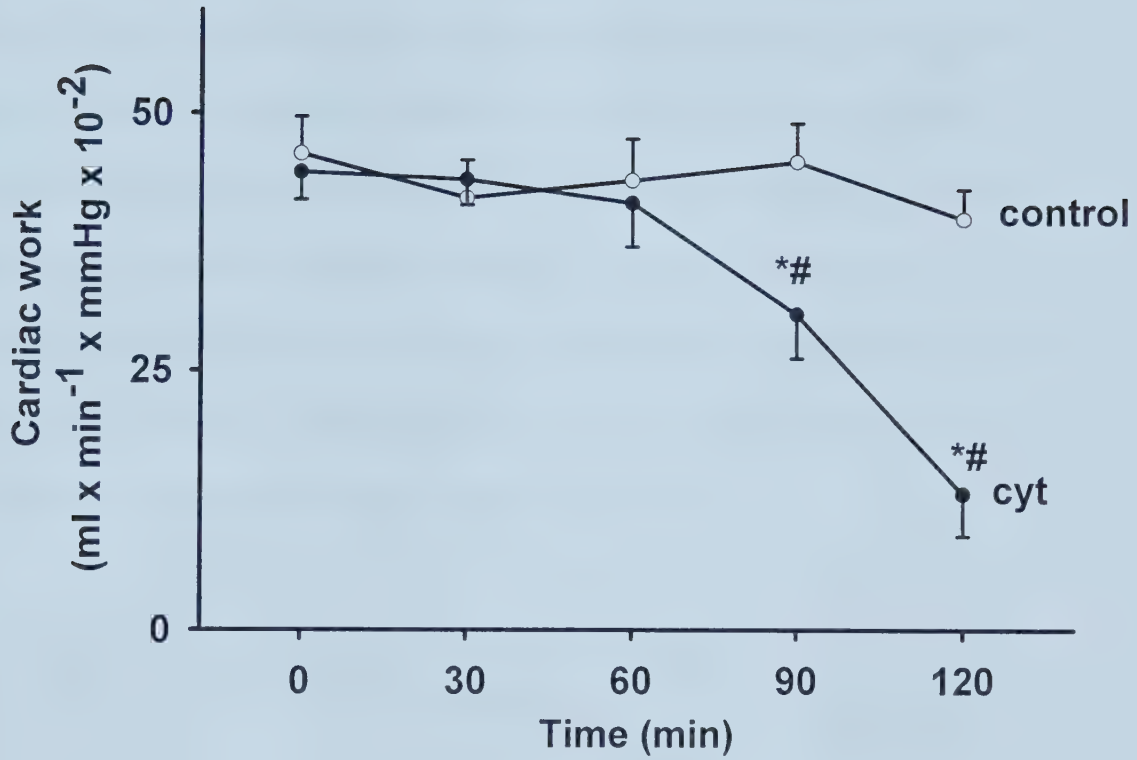

Figure 3.1. Time course of changes in cardiac work in control (n=7) and cytokine-treated (cyt, n=10) isolated working rat hearts. Cardiac work in control hearts remained stable for the first 90 min and declined slightly over the last 30 min whereas cytokine-treated hearts showed a significant loss in cardiac work between 60 and 120 min of perfusion.* $p<0.05$ versus 0 min within the same group (one way ANOVA); # $P<0.05$ versus control (two way repeated measures of ANOVA).

2. Effect of MMP-2 neutralizing antibody on cardiac function

Next it was determined whether MMP-2 antibody can protect the heart from pro-inflammatory cytokine-induced myocardial dysfunction. This MMP-2 antibody has been determined to be a MMP-2 neutralizing antibody as described[10]. Fig 3.2 shows the time course of mechanical function during 2 hr perfusion with MMP-2 neutralizing antibody or unrelated IgG control. MMP-2 antibody (30 μg/ml) abolished cytokine-induced myocardial depression whereas rat IgG exerted no significant protective effect. At the end of 2 hr perfusion, cardiac work (as a % of cardiac work at 0 hr) in cytokine + antibody treated group remained at 84 ± 5% (n=4) compared to 47 ± 3% (n=4) in the cytokine + IgG group. Changes in coronary flow are shown in Table 3.1.

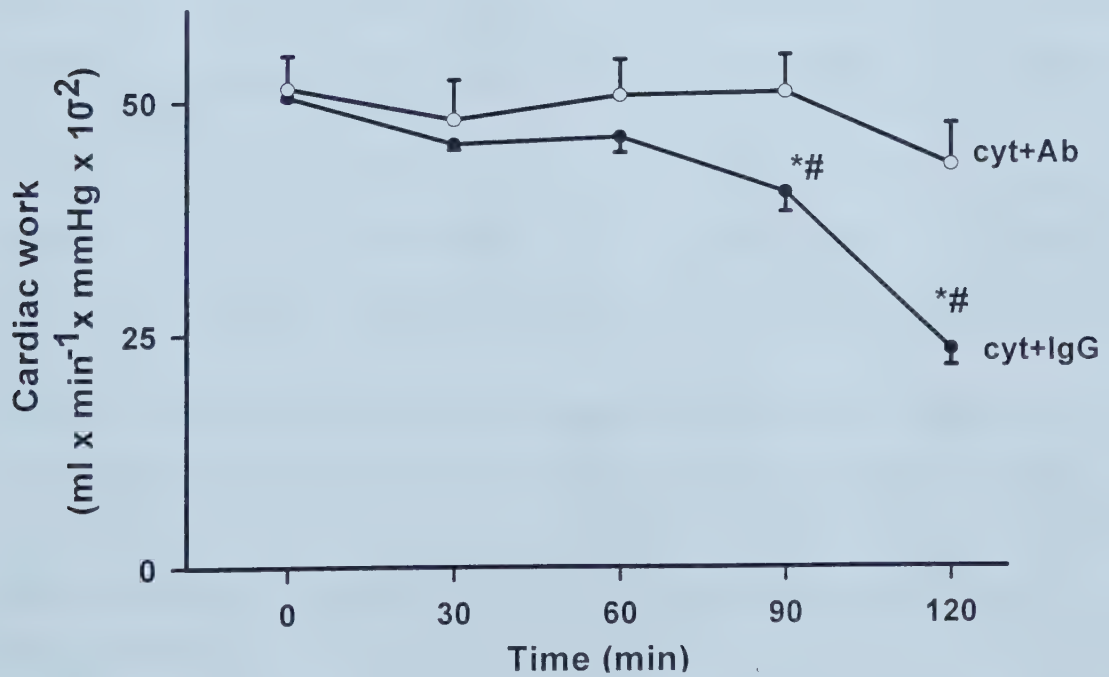

Figure 3.2 MMP-2 neutralizing antibody (30 μg/ml) abolished cytokine-induced myocardial depression, whereas unrelated IgG (30 μg/ml) exerted no significant protective effect. * P<0.05 versus 0 min within the same group (one way ANOVA); # P<0.05 versus cyt + IgG (two way repeated measures of ANOVA)

	peak systolic pressure mmHg	cardiac output ml/min	coronary flow ml/min
control (n=7)			
0 min	84.9 ± 1.5	54.1± 3.7	19.7 ± 1.4
60 min	85.1 ± 1.7	50.8 ± 3.8	20.3 ± 1.2
120 min	86.4 ± 2.5	45.9 ± 2.2	19.1 ± 0.9
cyt (n=10)			
0 min	87.1 ± 1.9	50.9 ± 3.0	21.2 ± 1.6
60 min	86.2 ± 2.3	47.6 ± 4.7	21.0 ± 1.8
120 min	59.4 ± 7.8*#	17.9 ± 4.6*#	11.0 ± 1.7*#
cyt + IgG (n=4)			
0 min	93.9 ± 5.0	54.3 ± 2.6	21.6 ± 2.6
60 min	90.1 ± 6.7	51.9 ± 2.0	24.2 ± 3.8
120 min	78.3 ± 7.1	31.0 ± 3.9*	26.8 ± 3.3
cyt + Ab (n=4)			
0 min	90.6 ± 1.9	57.1 ± 4.5	23.2 ± 2.5
60 min	87.2 ± 2.4	58.6 ± 4.9	23.4 ± 3.3
120 min	84.1± 2.9	51.7 ± 4.9#	25.2 ± 5.1

Table 3.1 Changes in peak systolic pressure, cardiac output, and coronary flow in control, cytokine, cytokine + IgG, cytokine + Ab groups. Values represent mean ± s.e.m., * $p < 0.05$ vs. respective baseline value at 0 min after 120 min perfusion (one way ANOVA). # $p < 0.05$ vs. corresponding control value (Student's t test).

3. Release of MMP-2 into perfusate during 2 hr perfusion

Figure 3.3 shows that isolated working rat heart perfused with recirculating buffer in the presence or absence of pro-inflammatory cytokines continuously release MMP-2 into the perfusate. Perfusate samples were taken at 1, 30, 60, 90, and 120 min in both groups and an extra time point -1 only in cytokine-treated hearts. The top panel is a representative zymogram showing the time course of changes in gelatinolytic activities from both control and cytokine-treated hearts, the function of which was shown in Fig 3.1. Gelatinolytic activities were detected at 75, 72, and 64 kDa corresponding to glycosylated pro-MMP-2, pro-MMP-2 and MMP-2 respectively, by comparison with the HT-1080 cell-derived standard. 72 kDa activity (pro-MMP-2) constituted the major gelatinase activity. In control hearts, there was a time-dependent accumulation of 72 kDa activity within the first hour of perfusion. Addition of cytokines markedly increased perfusate 72 kDa activity especially in the first 60 min of perfusion (Fig. 3.3, bottom panel). This preceded the onset of the delayed depression in cardiac work, which was first significantly impaired in the cytokine group at 90 min perfusion (Fig. 3.1).

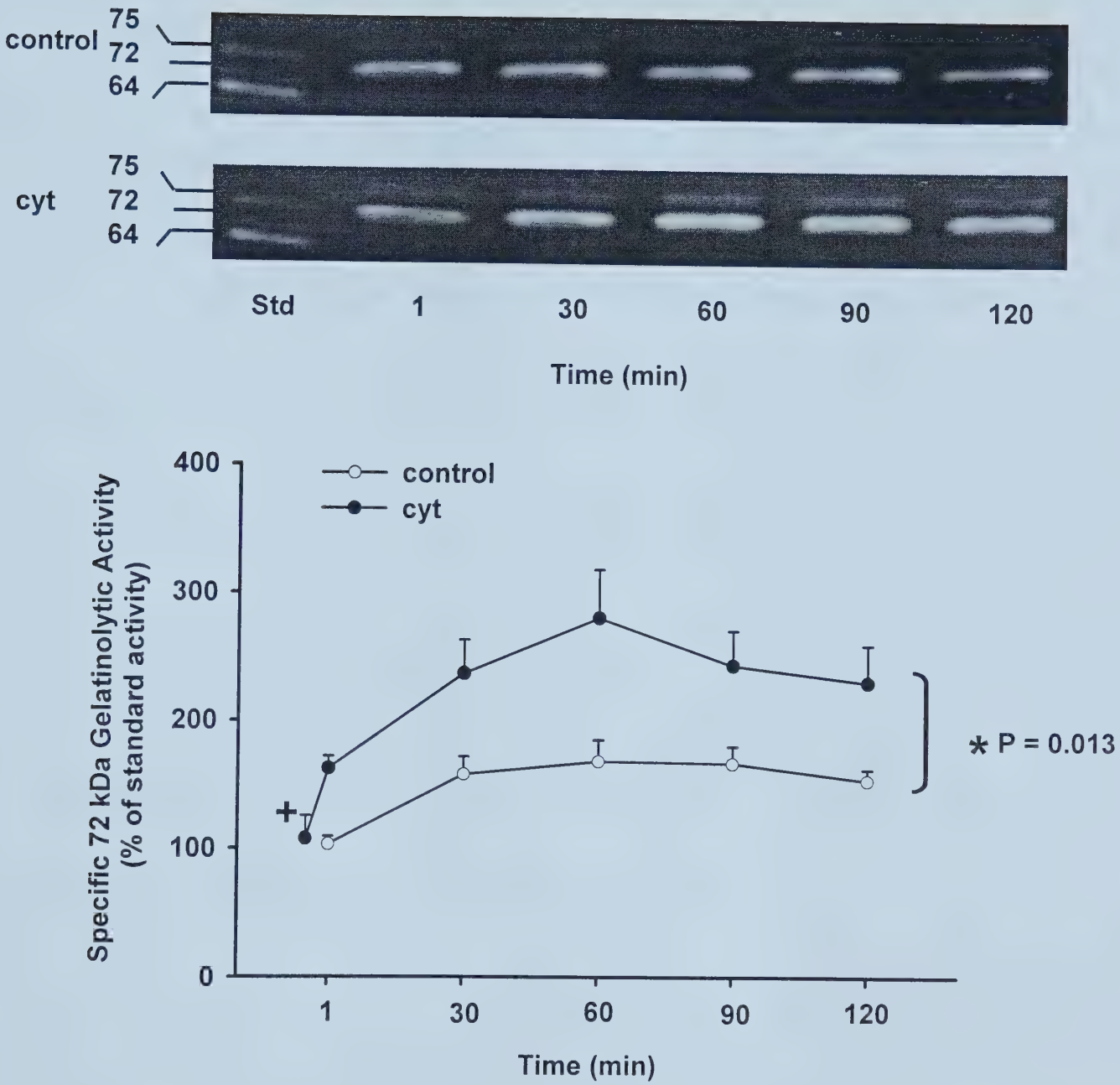

Figure 3.3 Release of MMPs into recirculating perfusate during 2 hr perfusion. Top panel: representative zymograms of perfusate samples from a control and a cytokine-treated (cyt) heart. Std indicates standard taken from HT-1080 cell conditioned medium. Bottom panel: densitometric analysis of specific 72 kDa gelatinolytic activity in perfusate from control (n=7) and cytokine-treated (n=10) hearts at 1, 30, 60, 90, and 120 min perfusion. One extra perfusate sample was taken 1 min before cytokine addition, denoted with + in cytokine-treated hearts. In control hearts there was a time-dependent accumulation of 72 kDa activity within the first hour of perfusion. Addition of cytokines markedly increased perfusate 72 kDa activity especially in the first 60 min of perfusion. * $P<0.05$, two-way repeated measures of ANOVA.

4. Changes of MMP activity in heart tissue

Effects of pro-inflammatory cytokines on myocardial MMP activity were determined by zymography using heart extracts taken after 2 hr perfusion. The top panel of Fig 3.4 is a representative tissue zymogram from a control (n=7), cytokine (n=10) and a cytokine plus MMP-2 neutralizing antibody treated (n=4) hearts. Tissue gelatinolytic activities showed a very similar profile as that seen in the perfusate samples (Fig 3.3). Specific 72 kDa gelatinolytic activity (pro-MMP-2) constituted the major gelatinase activity in all samples whereas 75 kDa and 64 kDa gelatinolytic activities were visible, they were not within the quantifiable range. Pro-MMP-9 and MMP-9 activities were not detected by zymography of heart tissue (data not shown). Concomitant with the enhanced release of 72 kDa activity into the perfusate, cytokines caused a significant 44% decrease in the heart tissue content at the end of 2 hr perfusion (Fig. 3.4). MMP-2 neutralizing antibody significantly attenuated the decline in tissue 72 kDa gelatinolytic activity caused by pro-inflammatory cytokines (see discussion).

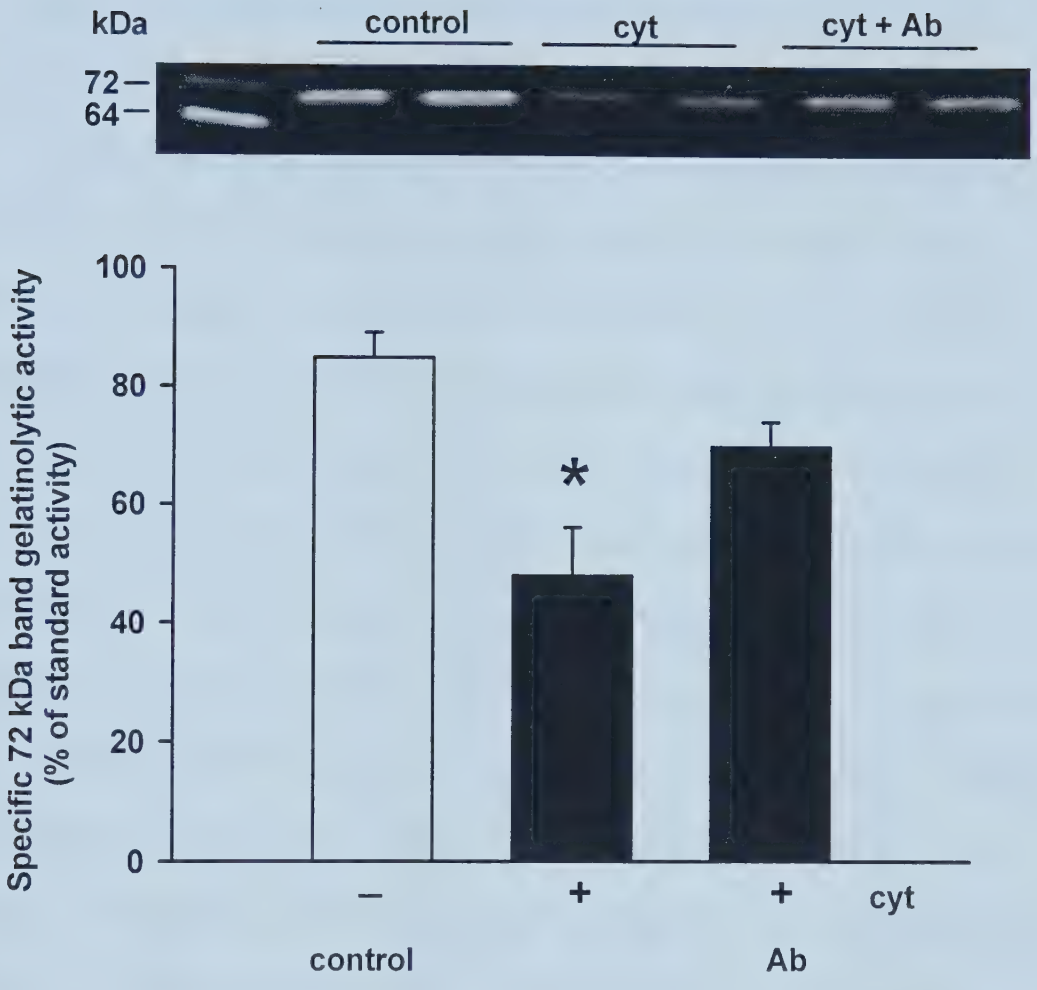

Figure 3.4 Top panel: representative zymogram of heart tissue samples from control (n=7), cytokine (cyt, n=10) or cytokine plus MMP-2 antibody (Ab, 30 μg/ml, n=4) treated hearts after 2 hr perfusion. Std denotes standard taken from HT1080 cell conditioned medium. Bottom panel: densitometric analysis of specific 72 kDa gelatinolytic activity shows that cytokines caused a significant decrease in heart tissue activity at the end of perfusion. Neutralizing MMP-2 antibody attenuated the decline in tissue 72 kDa activity caused by cytokines. * $P<0.05$ versus control (one-way ANOVA).

5. Effects of pro-inflammatory cytokines on myocardial NOS activity and perfusate dityrosine level

To confirm previous results from this laboratory that myocardial iNOS activity and peroxynitrite production were upregulated in the heart by pro-inflammatory cytokines[18], NOS activities (n=5 for each group) and the level of perfusate dityrosine (a marker of peroxynitrite) were measured in some hearts. The perfusate was supplemented with L-tyrosine (0.3 mM) which reacts with peroxynitrite to form nitrotyrosine and dityrosine, the latter which is detectable by fluorometry[119]. In ventricular tissue from hearts perfused for 120 min, the Ca^{2+}-dependent NOS activity (between 1-2 pmol/min/mg protein) was not significantly different between control and cytokine-treated hearts (Fig. 3.5, n=5). In contrast, cytokine treatment resulted in a marked increase in Ca^{2+}-independent activity, which was not abolished by MMP-2 neutralizing antibody. The level of perfusate dityrosine at 1, 30, 60, 90, and 120 min, showed time-dependent increase in control, cytokine, and cytokine + MMP-2 antibody groups ($P<0.05$ vs 1 min value, one way ANOVA). The elevation in perfusate dityrosine level in cytokine-treated hearts became significant at 90 min compared to control and remained so for the remaining perfusion time ($P<0.05$, Student's t test). Perfusate dityrosine level in cytokine + MMP-2 antibody-treated hearts had similar profile with cytokine-treated hearts, which became significantly elevated at 90 min compared to control (Fig 3.6).

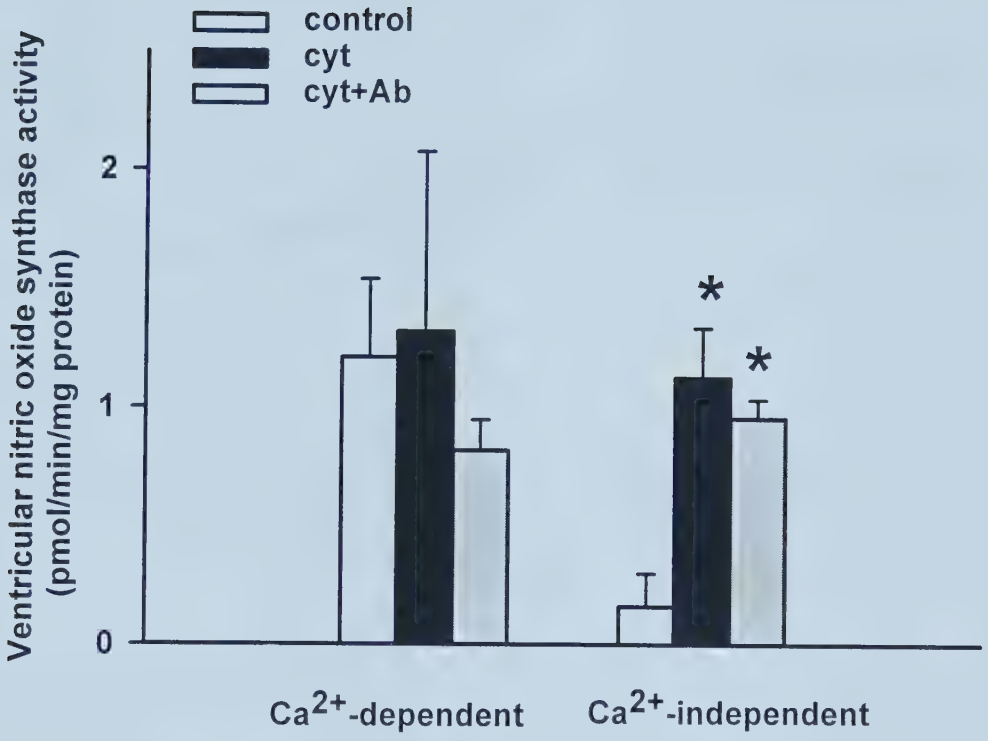

Figure 3.5 Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthase (NOS) activities in ventricular extracts from control, cytokine (cyt), and cytokine plus MMP-2 neutralizing antibody (Ab), treated hearts after 2 hr perfusion. There were no significant differences in Ca^{2+}-dependent activity. Cytokine treatment resulted in a 7-fold increase in Ca^{2+}-independent activity, which was unaffected by anti-MMP-2 antibody. * $P<0.05$ versus control (n=5 for each group, one way ANOVA).

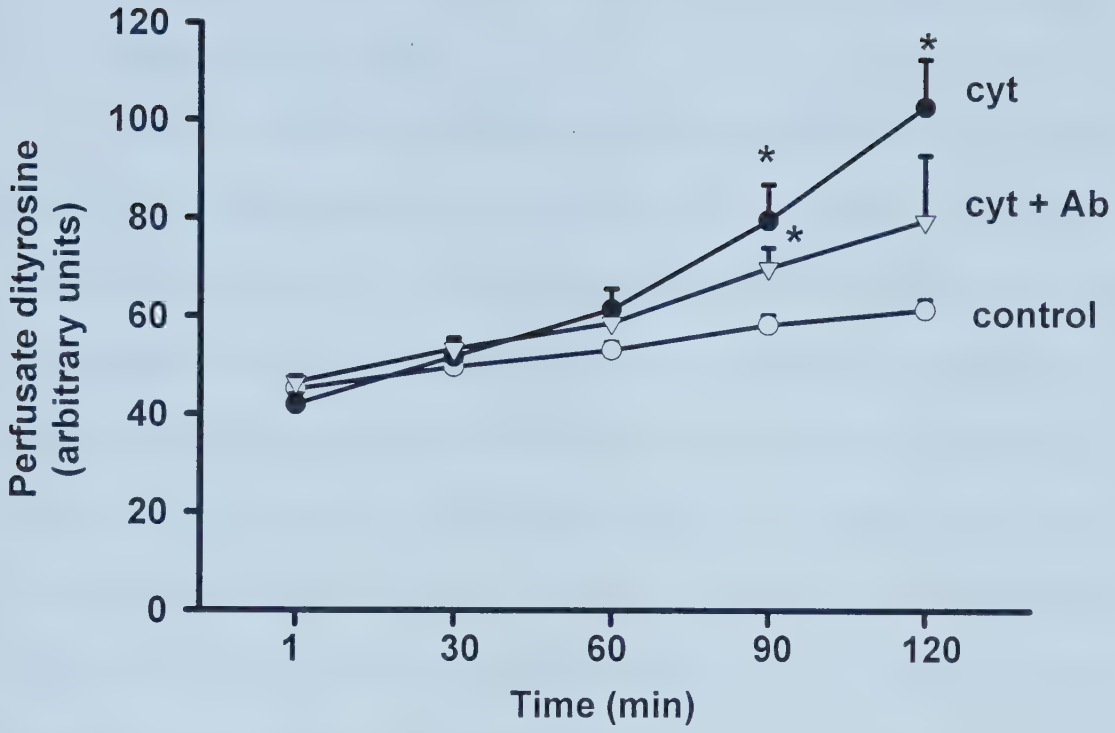

Figure 3.6 Time course of changes in perfusate dityrosine level in control (n=7) cytokine-treated (cyt, n=10), and cytokine + anti-MMP-2 antibody-treated hearts (cyt + Ab, n=4). The level of perfusate dityrosine in all three groups showed a time-dependent increase (One way repeated measures of ANOVA). Student's t test showed $P<0.05$ versus corresponding control value, denoted with *.

6. Effects of MMP inhibitors on functional recovery of cytokine-induced myocardial dysfunction

Next it was determined whether inhibitors of MMP activity can protect the heart from pro-inflammatory cytokine-induced myocardial dysfunction. MMP inhibitors PD166793 (0.2-10 μM) concentration-dependently inhibited the 72 kDa gelatinolytic activity of coronary effluent samples taken from an aerobically perfused heart whereas Ro31-9790 (0.01-1 μM) concentration-dependently inhibited the 72 kDa activity of HT 1080 cell conditioned medium (Figure 3.7). Figure 3.8 shows the time course of changes in cardiac work after the addition of Ro31-9790 (3 μM), PD166793 (2 μM) and DMSO vehicle (for Ro31-9790 and PD166793), or for comparison, anti-MMP-2 antibody (30 μg/ml) to cytokine-treated hearts. They all markedly attenuated the cytokine-induced loss in cardiac mechanical function. Expressed as a percentage of cardiac work compared to t = 0 min, at the end of 2 hr perfusion the percentage of cardiac work remaining for each group was (Fig 3.9): cytokine + DMSO vehicle, 30 ± 9% (n=4); cytokine + Ro31-9790, 70 ± 4% (n=6); cytokine + PD166796, 62 ± 5% (n=6); cytokine + MMP-2 antibody, 84 ± 5 % (n=4). Changes in coronary flow are shown in Table 3.2.

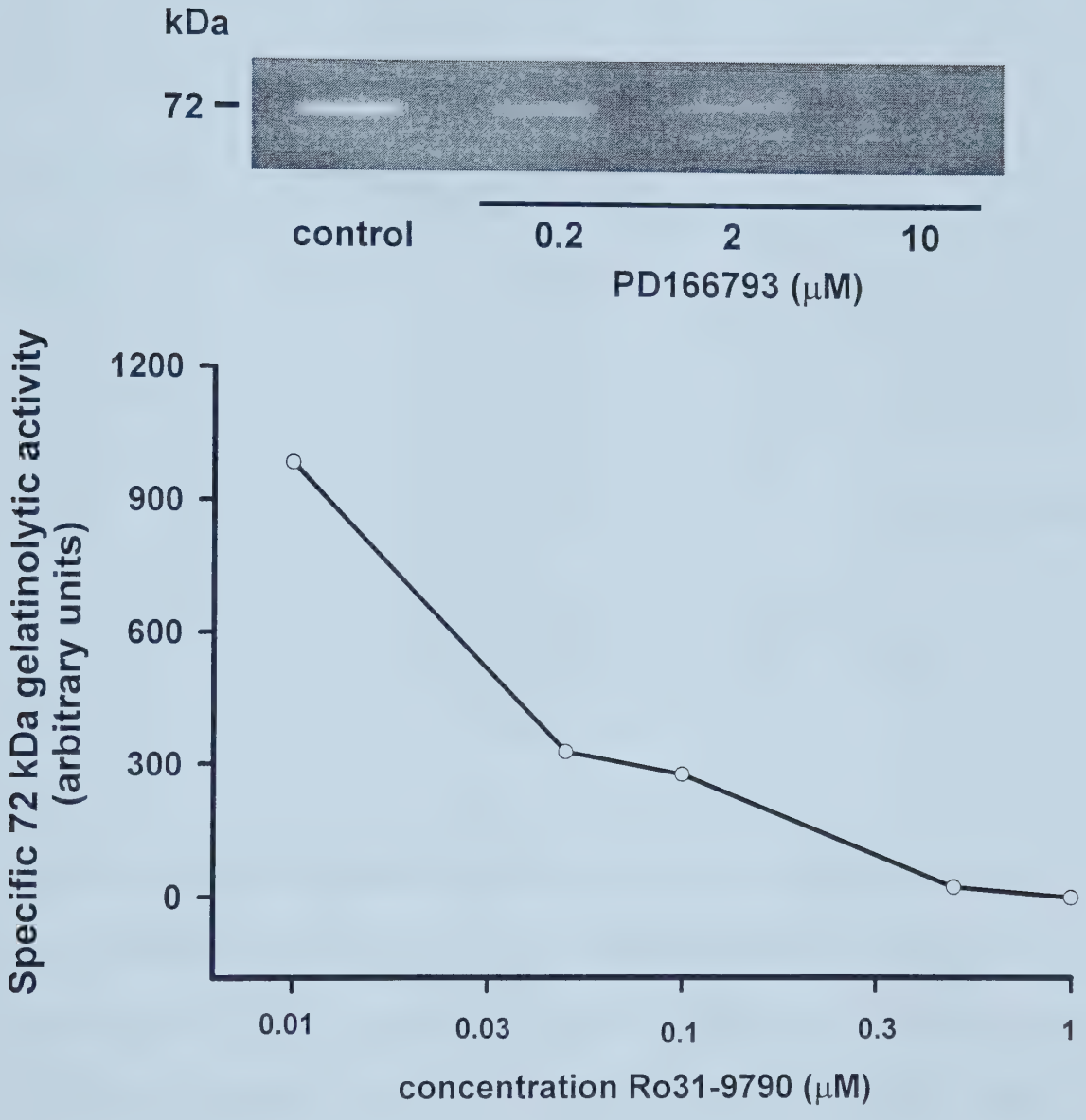

Figure 3.7 Inhibitory profile of PD166793 and Ro31-9790 on MMP activities. Top panel: PD166793, when added to the zymography incubation buffer, concentration-dependently inhibited the 72 kDa activity of coronary effluent sample from an aerobically perfused heart. Bottom panel: Ro31-9790 concentration-dependently inhibited 72 kDa gelatinolytic activity of HT 1080 cell conditioned medium.

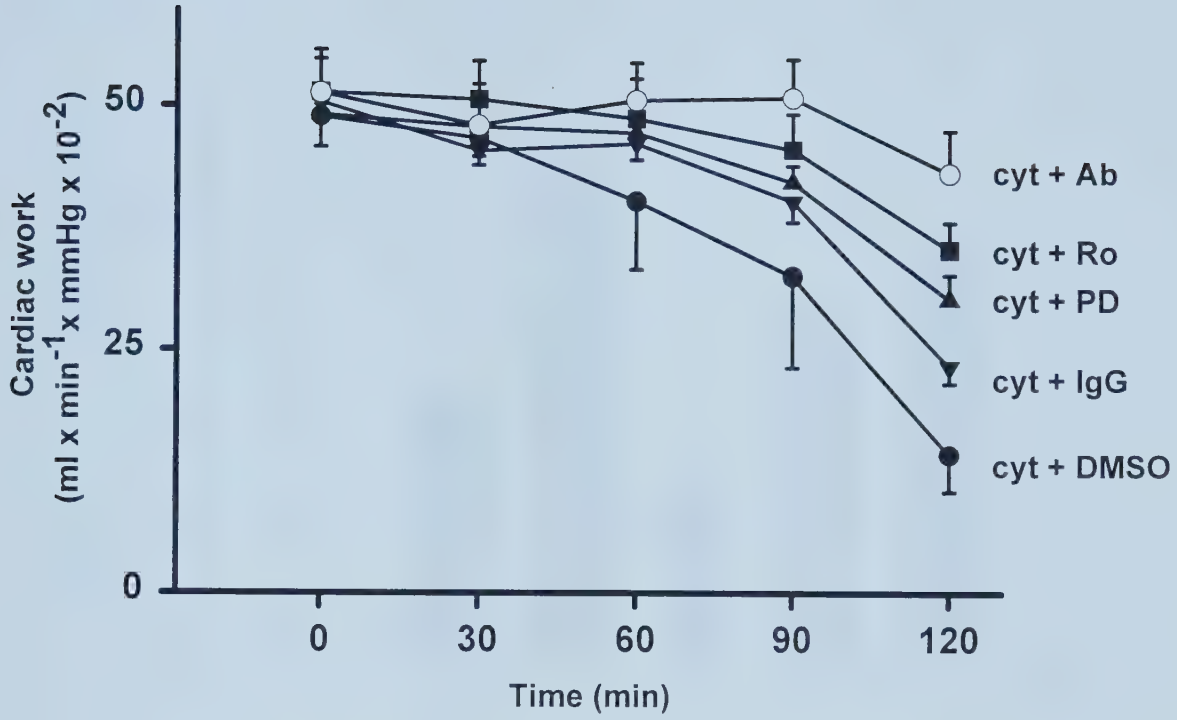

Figure 3.8 Effects of inhibition of MMPs on recovery of cardiac work during cytokine-induced myocardial dysfunction. Ro31-9790 (Ro, 2 μM, n=6), PD166793 (PD, 3 μM, n=6) and MMP-2 neutralizing antibody (Ab,30 μg/ml, n=4) significantly reduced the loss in cardiac work compared to cytokines + DMSO vehicle (n=4), cytokine + IgG (n=4), respectively. DMSO was the vehicle for Ro31-9790 and PD166793. For statistical analysis see Fig 3.9.

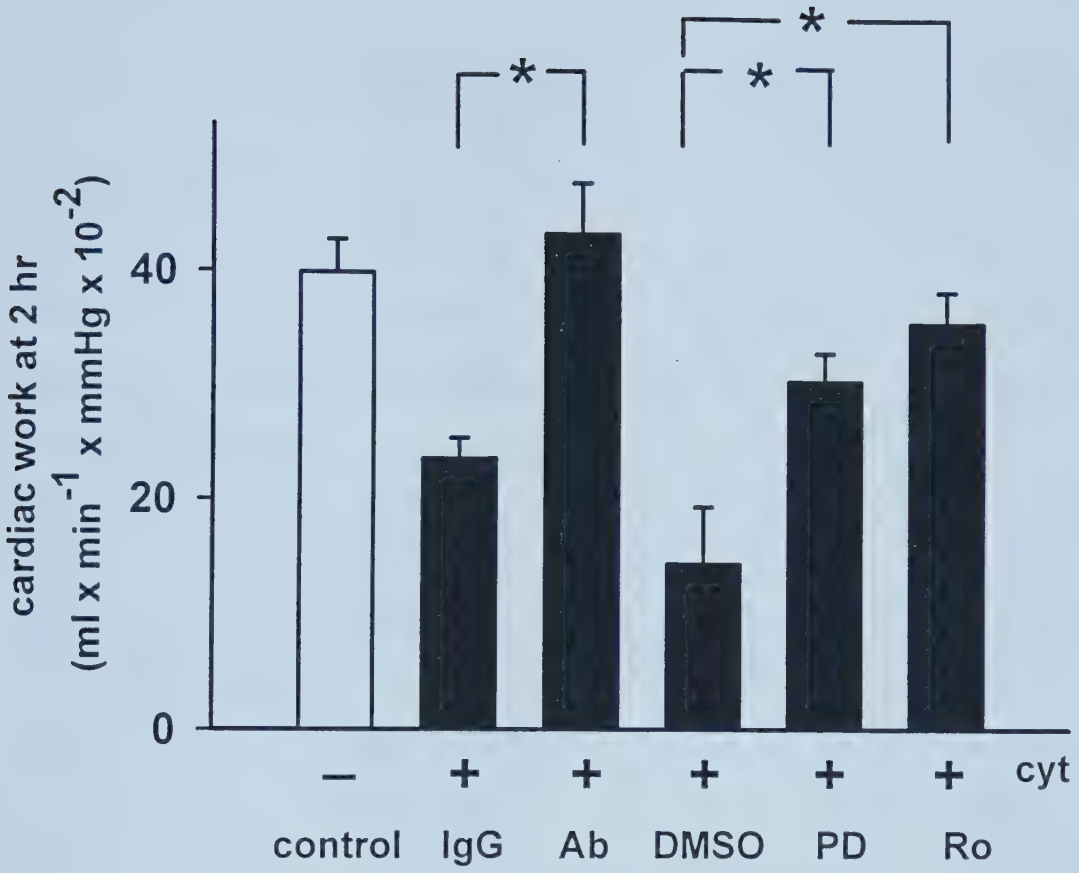

Figure 3.9 Cardiac work remaining at 2 hr. MMP-2 neutralizing antibody (Ab, 30 μg/ml) abolished cytokine-induced myocardial depression, whereas unrelated IgG (30 μg/ml) exerted no significant effect. MMP inhibitors (PD166793, 2 μM; Ro31-9790, 3 μM), but not DMSO vehicle, attenuated the cardiac depression caused by pro-inflammatory cytokines. * $P<0.05$ (one-way ANOVA, n=4-7 for each group).

	peak systolic pressure mmHg	cardiac output ml/min	coronary flow ml/min
DMSO (n=4)			
0 min	90.3 ± 3.3	54.7 ± 4.6	24.1 ± 3.5
60 min	82.2 ± 6.2	48.9 ± 7.6	23.7 ± 2.2
120 min	59.8 ± 9.5*	23.1 ± 4.1*	15.6 ± 3.8
cyt + Ro (3 μM, n=6)			
0 min	88.2 ± 2.4	50.0 ± 3.7	26.2 ± 3.7
60 min	84.1 ± 2.6	49.6 ± 3.0	24.9 ± 3.6
120 min	77.0 ± 1.9*	39.5 ± 2.7#	24.4 ± 1.7#
cyt + PD (2 μM, n=6)			
0 min	89.1 ± 1.0	55.4 ± 1.7	25.9 ± 2.3
60 min	85.6 ± 1.2	55.6 ± 2.3	24.3 ± 2.1
120 min	77.1 ± 1.2*#	39.5 ± 3.1*#	23.8 ± 2.3#

Table 3.2 Changes in peak systolic pressure, cardiac output, and coronary flow in cytokine + DMSO, cytokine + Ro (3 μM), cytokine + PD (2 μM) groups. Values represent mean ± s.e.m., * $p < 0.05$ vs. respective baseline value at 0 min of 120 min perfusion (one way ANOVA). # $p < 0.05$ vs. DMSO vehicle control (Student's t test).

7. Changes in myocardial TIMP-4 in cytokine-treated hearts

Since the balance between MMPs and TIMPs might be altered in an inflammatory environment, and TIMP-4 is preferentially expressed in the heart[26], I then determined TIMP-4 protein expression in the heart by western blot analysis. TIMP-4 levels in ventricular extracts were expressed as percentage of a TIMP-4 standard obtained from rat myocardium. To compare to the effect of 2 hr in vitro working heart perfusion an extra set of hearts were perfused as Langendorff hearts for 15 minutes. There was no significant difference in TIMP-4 levels between these hearts and the control working hearts perfused for 2 hr (Fig 3.10). TIMP-4 was detected in control working hearts perfused for 2 hr and was significantly decreased by 51% in cytokine-treated hearts (Fig 3.10).

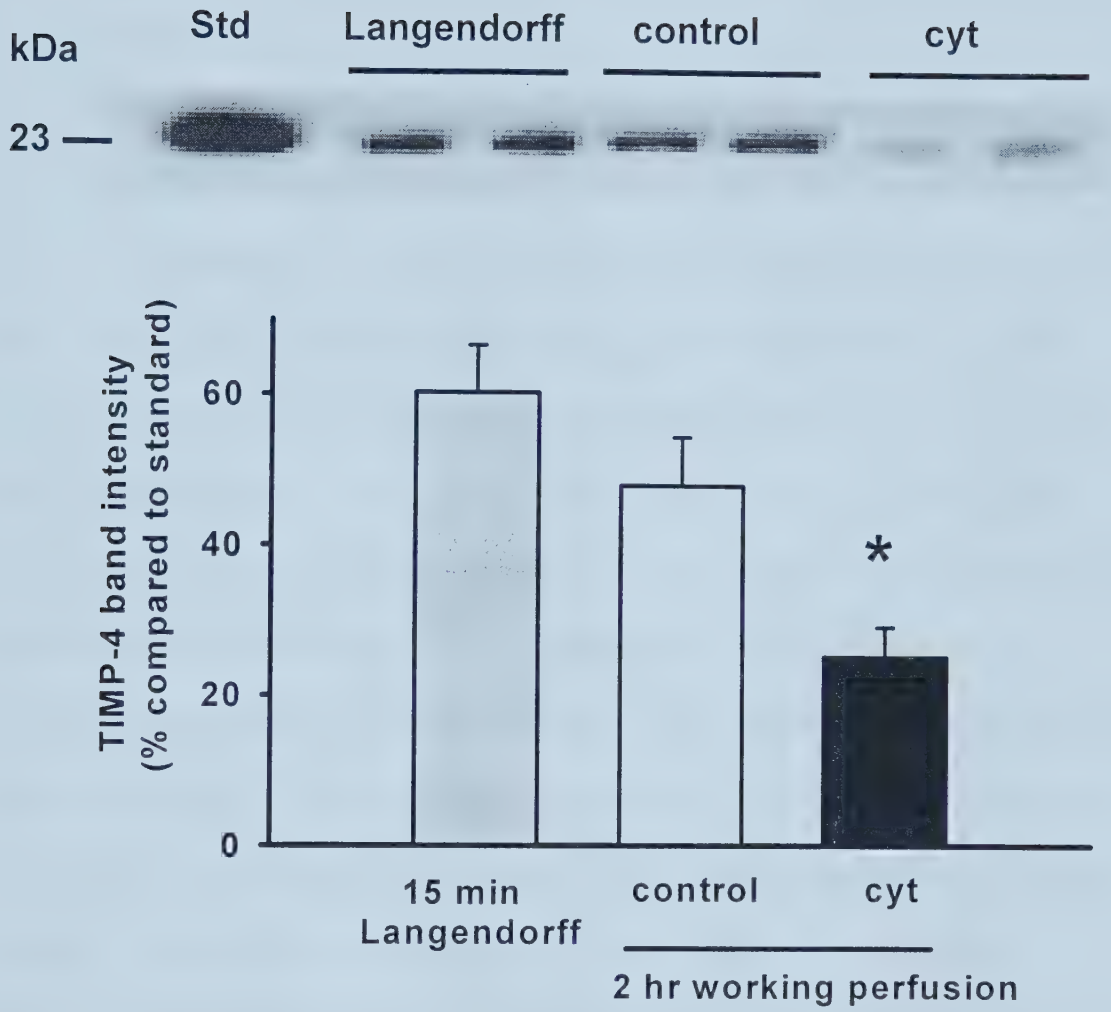

Figure 3.10 Top panel: Representative western blot of TIMP-4 in ventricular extracts from hearts perfused for 15 min in Langendorff mode or, control and cytokine-treated (cyt) working rat hearts perfused for 120 min. Std denotes rat myocardial TIMP-4 standard. Bottom panel: Densitometric analysis of TIMP-4 levels. TIMP-4 was significantly decreased in cytokine-treated hearts compared to control and to 15 min Langendorff perfused hearts. * $P<0.05$ (One-way ANOVA, n=5 in each group).

B. Scavenging $ONOO^-$ attenuates cytokine-induced myocardial dysfunction

1. Effect of pro-inflammtory cytokines on cardiac mechanical function in spontaneously beating hearts

The combination of three pro-inflammatory cytokines (5 ng/ml IL-1β, 20 ng/ml TNF-α, and 9 ng/ml IFN-γ), was investigated in spontaneously beating isolated working hearts. Changes in cardiac mechanical function showed a very similar profile (Figure 3.11) as did the paced hearts (Fig 3.1). Cardiac work (cardiac output X peak systolic pressure) in control hearts remained stable for the first 90 min of perfusion, followed by a gradual decline over the last 10 min ($P<0.05$, one-way ANOVA). Cytokine-treated hearts remained stable for the first 60 min of perfusion, and then declined significantly in cardiac work between 60 and 120 min of perfusion to 35% of their original cardiac work ($P<0.05$, one-way ANOVA). The depression in cardiac work in cytokine-treated hearts was significantly different from the loss in cardiac work in control hearts ($P<0.05$, two-way repeated measures of ANOVA). NO difference in heart rate between groups was observed (data not shown). Changes of coronary flow were shown in Table 3.3. These results suggest that pro-inflammatory cytokines also cause myocardial dysfunction in spontaneously beating hearts.

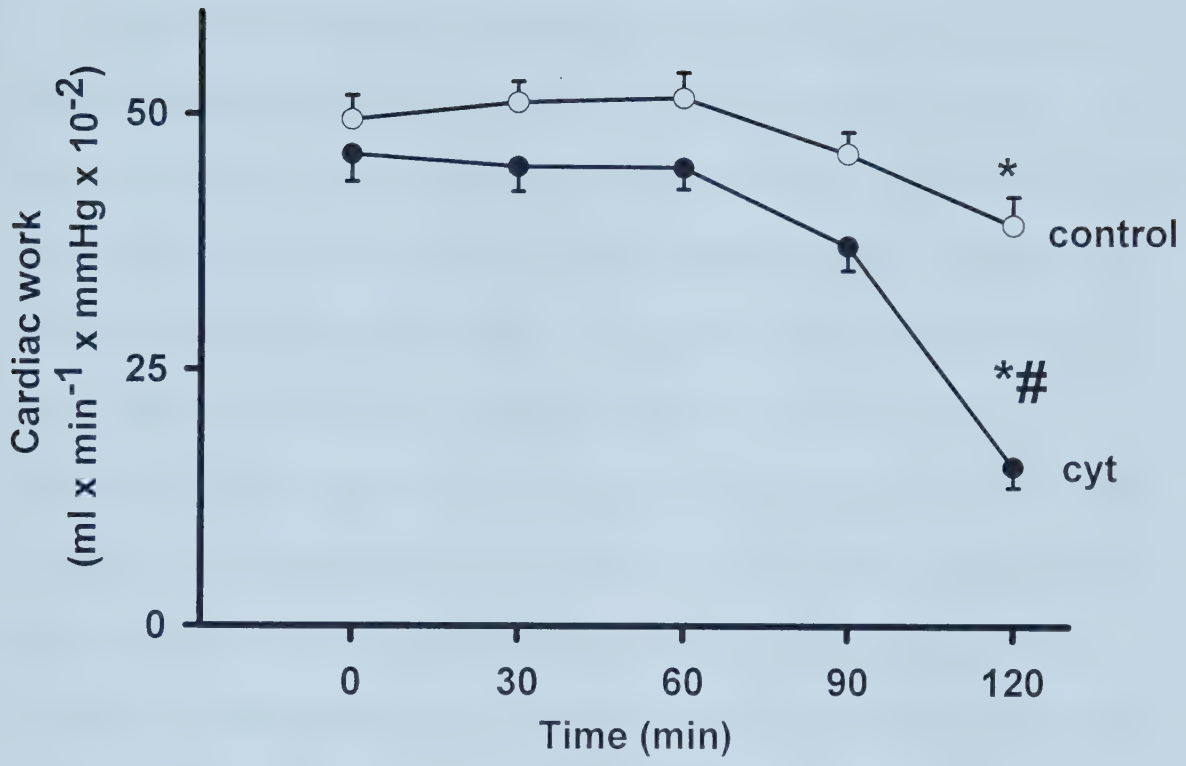

Figure 3.11 Time course of changes in cardiac work in control (n=6) and cytokine-treated (n=7) isolated working hearts which were spontaneously beating. Cardiac work in control hearts remained stable for the first 90 min and declined slightly over the last 30 min whereas cytokine-treated hearts showed a significant loss in cardiac work between 60 and 120 min of perfusion. * $P<0.05$ versus 0 min within the same group (one way ANOVA); # $P<0.05$ versus control (two way repeated measures of ANOVA).

2. Effect of $ONOO^-$ scavenger on cardiac mechanical function

As cytokine treatment was previously shown to enhance the production of NO, superoxide, and peroxynitrite with a concomitant decline in cardiac mechanical function[18], we then tested the effects of a peroxynitrite decomposition catalyst, FeTPPs and its inactive form, TPPs. Additional series of hearts were perfused in the presence of cytokines with 50 μM FeTPPs, or its inactive form TPPs (50 μM). It was previous shown that 50 μM FeTPPs alone did not significantly change myocardial function over 120 minutes of perfusion[18]. FeTPPs prevented the cytokine-induced loss in cardiac work (Fig 3.12). Although TPPs appeared to have an intermediate effect, this was not statistically significant compared to cytokine-treated hearts. No difference in heart rate between groups was observed (data not shown). Changes of coronary flow are shown in Table 3.3.

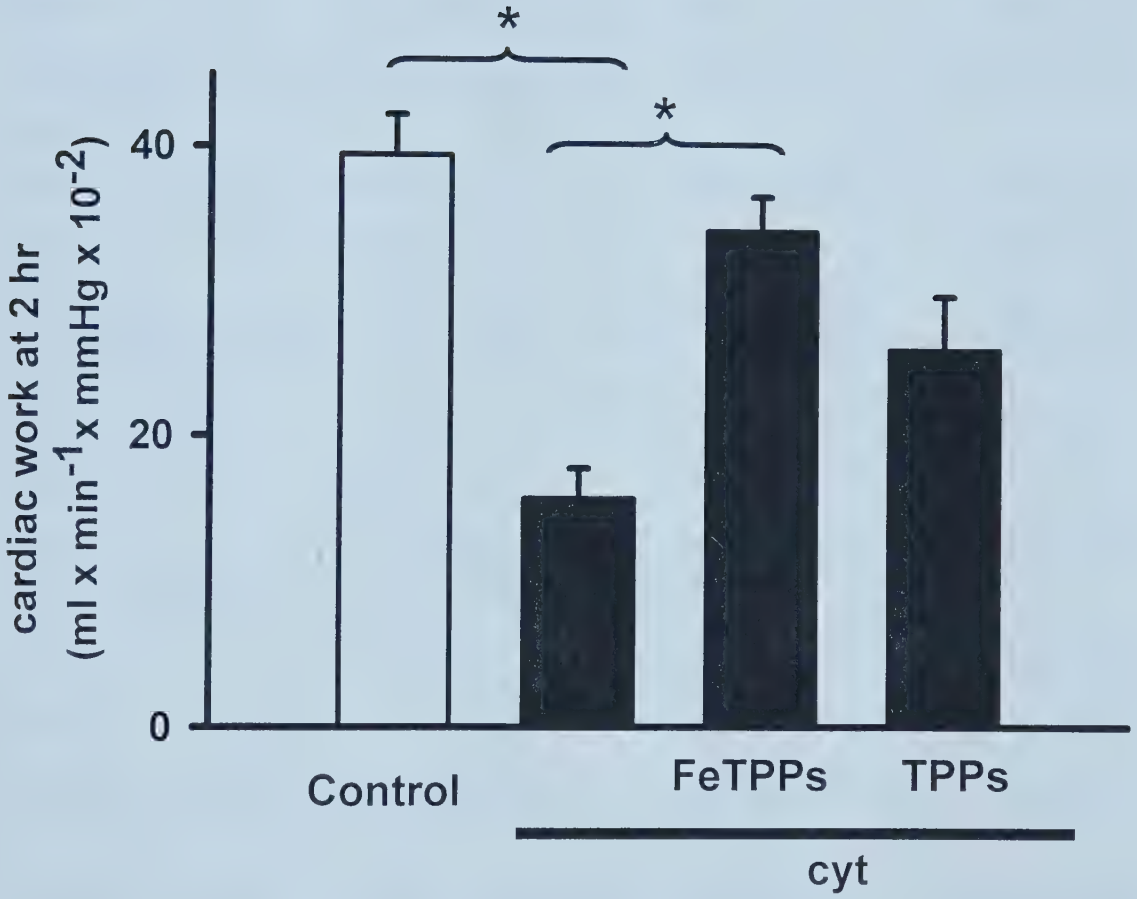

Figure 3.12 Effects of peroxynitrite scavenger on recovery of cardiac work during cytokine-induced myocardial dysfunction. At the end of 2 hr perfusion, FeTPPs (50 μM, n=6) abolished the depression of cardiac work caused by pro-inflammatory cytokines, while its inactive form TPPs (50 μM, n=6) plus cytokine did not have a significant protective effect. * $P<0.05$ (one way ANOVA).

	peak systolic pressure mmHg	cardiac output ml/min	coronary flow ml/min
control (n=6)			
0 min	93.2 ± 3.3	53.3 ± 2.2	22.3 ± 1.8
60 min	91.8 ± 3.8	56.4 ± 0.7	23.6 ± 2.3
120 min	85.7 ± 3.5	45.2 ± 2.8*	24.4 ± 2.8
cyt (n=7)			
0 min	92.9 ± 2.7	49.8 ± 2.9	19.0 ± 1.6
60 min	88.9 ± 3.2	50.6 ± 1.9	20.6 ± 2.1
120 min	72.1± 4.1*#	21.2 ± 2.1*#	15.9 ± 1.1#
cyt + FeTPPs (n=6)			
0 min	89.1 ± 1.3	56.8 ± 1.1	25.2 ± 1.9
60 min	86.7 ± 1.3	56.7 ± 0.9	24.4 ± 1.1
120 min	78.3 ± 2.0	43.6 ± 2.4*	26.2 ± 1.8
cyt + TPPs (n=6)			
0 min	91.2 ± 1.9	53.9 ± 1.5	22.8 ± 2.0
60 min	88.1 ± 2.2	51.6 ± 2.0	22.4 ± 2.2
120 min	75.6 ± 3.4*	33.9 ± 4.0*	23.2 ± 3.3

Table 3.3 Changes in peak systolic pressure, cardiac output, and coronary flow in control, cytokine, cytokine + FeTPPs, and cytokine + TPPs groups. Values represent mean ± s.e.m., * $p < 0.05$ vs. respective baseline value at 0 min of 120 min perfusion (one way ANOVA). # $p < 0.05$ vs. corresponding control value (Student's t test).

3. Effects of FeTPPs and TPPs on MMP activity in vitro

In order to determine whether FeTPPs had an inhibitory effect on the gelatinolytic activities of MMPs in addition to its $ONOO^-$ scavenging effect, FeTPPs (0-50 μM) or TPPs (0-50 μM) were added to the zymography incubation buffer during the overnight incubation of purified human pro-MMP-2 (Figure 3.13). Both FeTPPs and TPPs concentration-dependently inhibited the 72 kDa activity ($P<0.05$, one way ANOVA, n=4 for each group). FeTPPs was more potent in inhibiting 72 kDa activity than TPPs, especially seen at the higher concentrations of 30 and 50 μM (Figure 3.13). The IC_{50} of FeTPPs (14.7 ± 1.5) μM was significantly lower than that of TPPs (22.2 ± 4.4 μM, $P<0.05$, Student's t test, n=4 for each group).

4. Time course of myocardial MMP activity

In order to determine the time course of changes in myocardial 72 kDa gelatinolytic activity and to compare the loss of cardiac work with the loss of gelatinolytic activity, a separate series of spontaneously beating hearts were perfused for either 0, 30, 60, or 120 minutes in the absence (control) or presence of 5 ng/ml IL-1β, 20 ng/ml TNF-α, and 9 ng/ml IFN-γ combined (cytokine-treated). The ventricles were frozen and processed as described[17]. Both control and cytokine-treated hearts showed a time-dependent decrease in 72 kDa gelatinolytic activity (Figure 3.14, $P<0.05$, one way ANOVA, n=5-8 for each group). However, cytokine treatment accelerated the loss of tissue 72 kDa gelatinolytic activity at 60 and 120 minutes compared to control hearts ($P<0.05$, Student's t test, n=5-8 for each group).

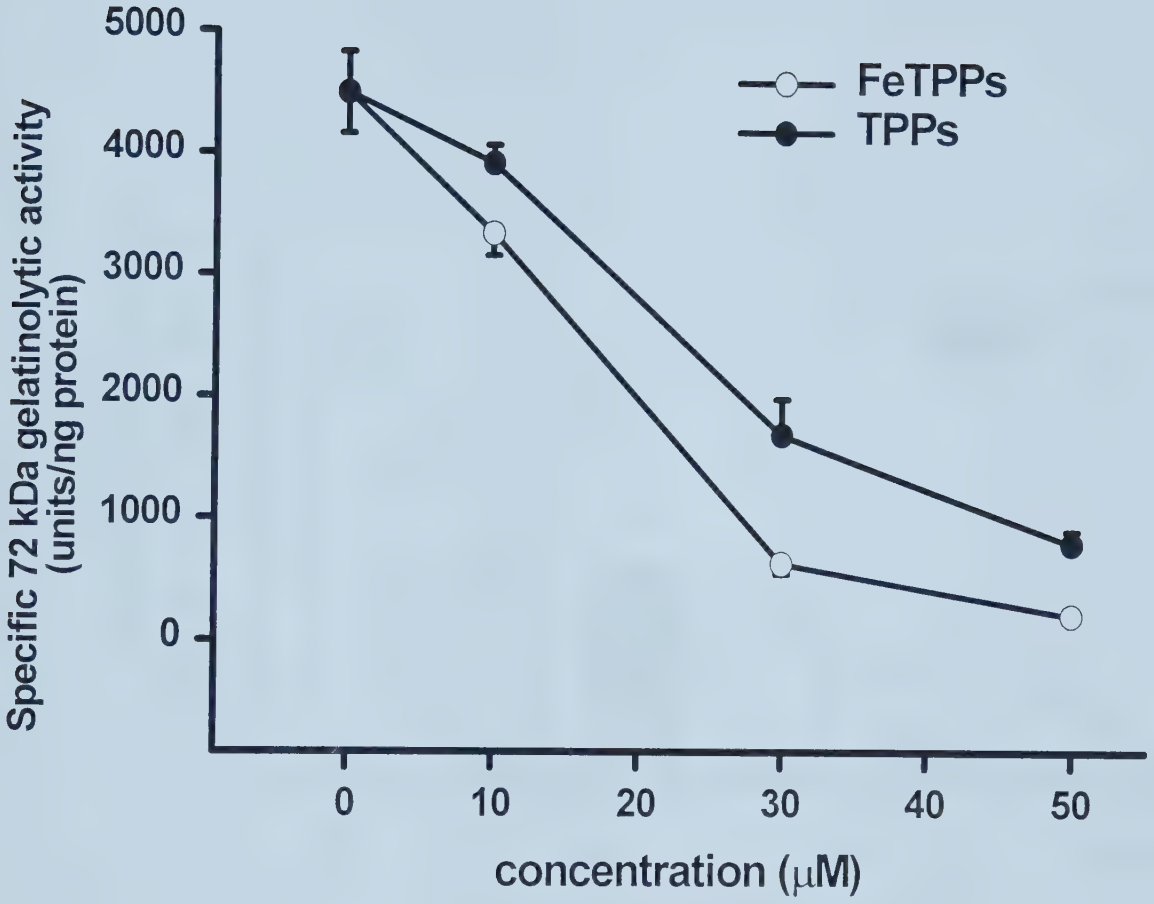

Figure 3.13 Inhibitory effects of FeTPPs and TPPs on purified human pro-MMP-2 (72 kDa gelatinolytic activity) in vitro (n=4 for each group). Both FeTPPs and TPPs concentration-dependently inhibited 72 kDa activity ($P<0.05$, one way ANOVA). FeTPPs (IC_{50} = 14.7 ± 1.5 μM) was more potent than TPPs (IC_{50} = 22.2 ± 4.4 μM) ($P<0.05$, Student's t test, n=4 for each group).

.

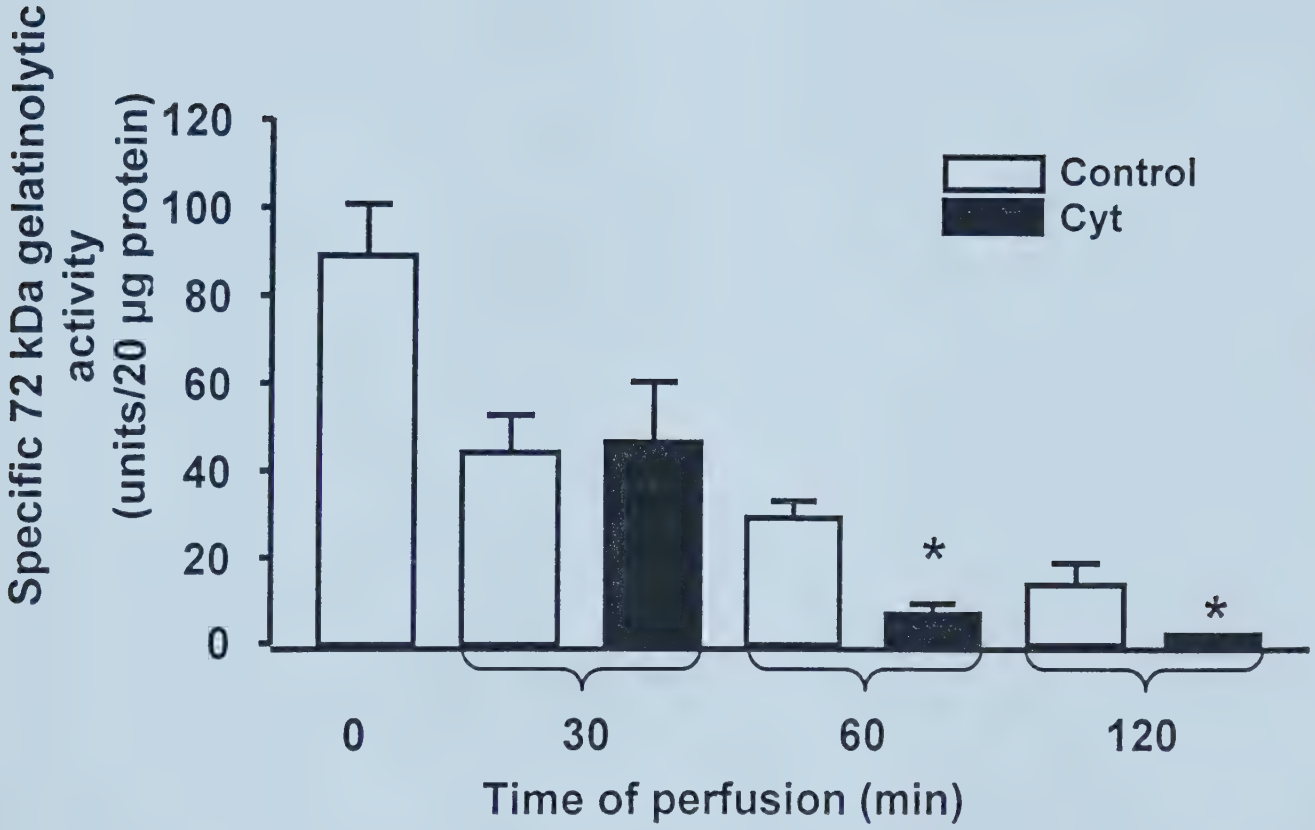

Figure 3.14 Time course of changes in 72 kDa gelatinolytic activity in ventricular extracts from control and cytokine-treated (cyt) working rat hearts. Both control and cytokine-treated groups showed a time-dependent loss of 72 kDa gelatinolytic activity. There is an accelerated loss of tissue 72 kDa gelatinolytic activity in cytokine-treated hearts between 60 and 120 min perfusion. * $P<0.05$ vs control (Student's t test, n=5-8 for each group).

CHAPTER IV

DISCUSSION

A. Inhibition of MMP activity study

I have shown here for the first time that pro-inflammatory cytokines cause a rapid and enhanced release of pro-MMP-2 activity from the heart into the perfusate. This preceded the onset of a marked depression in cardiac mechanical function. At the end of 2 hr perfusion both the remaining pro-MMP-2 activity and the level of its endogenous inhibitor, TIMP-4, in heart tissue were reduced in cytokine-treated hearts. Pharmacological inhibitors of MMPs or a neutralizing MMP-2 antibody protected hearts from the cytokine-induced myocardial dysfunction.

This laboratory has recently shown using this model of acute myocardial contractile dysfunction that pro-inflammatory cytokines enhance the myocardial generation of the pro-oxidant species peroxynitrite through an enhancement in both superoxide and NO generating enzyme activities, which are together responsible for the depression in contractile function[18]. This was confirmed in the present study with the enhancement in both inducible NO synthase activity in heart tissue and in the level of perfusate dityrosine, an indicator of peroxynitrite generation[110,119].

This laboratory has also recently found that the activation and release of MMP-2 from the heart contributes to another type of oxidant stress induced injury to the heart caused by ischemia and reperfusion[10]. The oxidant stress in ischemia-reperfusion injury is also caused by the generation of peroxynitrite[110,119]. As MMPs can be activated by oxidant stress[103], I determined

here whether cytokine-induced cardiac dysfunction is also mediated through MMPs.

In accordance with a previous report from this laboratory[10], the main gelatinolytic activity in coronary effluent from the aerobically perfused heart is 72 kDa, corresponding to proMMP-2. MMP-2 is expressed in endothelial and endocardial cells, sub-endocardial layers[120] as well as in cardiac myocytes[12]. A rapid and significant increase in 72 kDa activity was observed within the first hour in the perfusate from hearts exposed to cytokines along with release of 64 kDa activity corresponding to MMP-2, albeit at a reduced level. I did identify MMP-9 (gelatinase B) activity in the coronary effluent, but not in the heart tissue. It may due to tight combination between MMP-9 and TIMP-1 in heart tissue. In addition, I also found an unidentified gelatinase at 75 kDa. This 75 kDa gelatinase may be a glycosylated form of MMP-2 or may be an activated form of MMP-9 by neutrophil elastase[21]. The significance of this form is not known. Moreover, the enhanced release of proMMP-2 in the perfusate and a clear-cut depletion of pro-MMP-2 activity in the myocardium associated with the impaired cardiac mechanical function suggested a role of MMP-2 in cytokine-induced myocardial dysfunction.

Pro-MMPs may be activated by breaking a zinc-cysteine bond[65], which exposes its catalytic site followed by proteolytic activation, or through oxidant-induced conformational changes[69]. Previous study suggested that oxygen free radicals may be capable of activating matrix metalloproteinases in perfused rat hearts[49]. Indeed, the powerful oxidant peroxynitrite can activate pro-MMP-2 in

human smooth muscle cells[80] and 1-20 μM peroxynitrite activated purified proMMP-8 without a change in molecular weight[69]. It may be therefore more accurate to specify the MMP-2 activities reported here by molecular weight than by the designations "proMMP-2" and "MMP-2" which imply inactive and active forms of the enzyme, respectively, when this indeed may not be the case. It has been shown that pro-inflammatory cytokines, via concerted stimulation of cardiac iNOS, xanthine oxidase, and NADH oxidase activities, enhance the formation of NO and superoxide, and their reaction product, $ONOO^-$[18]. Whether there is an action of $ONOO^-$ on the release of MMP-2 is not known, it is possible that $ONOO^-$ activates MMP-2 which then may contribute to decreased myocardial function. Endogenous tissue inhibitors of metalloproteinases (TIMPs) also control MMP activity. Interestingly, Frears et al demonstrated the inactivation of a tissue inhibitor of MMPs by $ONOO^-$ in vitro[23]. Since TIMP-4 is abundant in the heart[27] , we also investigated the changes in TIMP-4 protein levels. There was also a decrease in the level of TIMP-4 levels resulting from the exposure of the heart to cytokines. I was unable to measure myocardial TIMP activity by reverse zymography because of the multiple proteins in the heart tissue. It is interesting to speculate that this loss in TIMP-4 protein also contributed to enhanced MMP activity and the depression in myocardial function.

Compared to our results, the changes of MMP activity and TIMP level has been shown to occur in pathological alterations of myocardial ischemia[10], infarct[105], and dilated cardiomyopathies[46]. Li et al. demonstrated that TIMP-1 and –3 were downregulated at both mRNA and protein levels in the failing human

heart. In contrast, only TIMP-4 protein was selectively downregulated and myocardial MMP-9 gelatinolytic activity was enhanced in ischemic cardiomyopathy[46]. The same group also showed that in neonatal cardiac myocytes subjected to pro-inflammatory cytokines TNF-α and IL-1β, TIMP-3 was robustly decreased at both mRNA and protein levels along with upregulation of gelatinolytic activity. Transient increase in TIMP-4 and no change of TIMP-2 was also observed[47]. The cytokines induced downregulation of TIMPs and upregulation of metalloproteinases in the failing human heart. However some discrepancies do exist, such as the down regulation of TIMP-4 protein in ischemic failing human heart[46] and in our hand, both MMP activities and TIMP-4 level decreased in rat myocardium. It should be noted that studies of the failing heart are complex. The results could be affected by the end stage of the disease, hemodynamic variations, different pharmacological agents and pleiotropic effect of pro-infammatory cytokines. Baghelai et al. demonstrated a significant decrease in TIMP-1 gene expression while MMP-1 mRNA expression was unchanged in globally stunned rabbit hearts[3]. Cleutjen et al. found increased in MMP-1, MMP-2, and MMP-9 activities in infarcted left ventricle, which peaked at day 7 and declined thereafter[11]. Although types of MMPs and TIMPs were differently regulated in different heart disease, there is no doubt that the changes between MMPs and TIMPs in the cell could contribute to the pathology of myocardial dysfunction.

Therefore, agents that restore the TIMP-MMP equilibrium in cardiac tissue might have clinical relevance. Recently, inhibitors of matrix

metalloproteinase such as recombinant TIMP or synthetic inhibitors including some antibiotics with MMP inhibitory activity (tetracyclines, doxycycline, minocycline) turned out to be an effective therapeutic tools in treating failing hearts. Indeed, the results from recent studies suggest that administration of synthetic matrix metalloproteinase inhibitors improves left ventricular geometry and pump function, and blocks the progression of congestive heart failure. Rohde et al. demonstrated that MMP inhibition attenuated early left ventricular enlargement after experimental myocardial infarction in mice[81]. Cheung et al. showed that MMP inhibitors or neutralizing anti-MMP-2 antibody improved the recovery of mechanical function in isolated rat hearts subjected to ischemia and reperfusion[10]. In the setting of the pro-inflammatory cytokine exposed heart, I showed that inhibition of MMPs is beneficial to preserve cardiac mechanical function. An in vivo study showed that MMP inhibition abrogated endotoxin induced lethality and prevented hepatic injury in endotoxemic mice[63]. Currently, six MMP inhibitors are believed to be in clinical trials in patients with corneal ulcers, lung cancer, inflammatory diseases, etc. It is therefore feasible that such treatment may be used in the future for other clinical applications such as cardiovascular disease. More research is needed to determine the specificity, safety, toxicity, bioavailability and efficacy of these drugs.

In addition to inhibitor-based approaches, transgenic techniques have opened new and promising avenues for the study of MMP function in diverse myocardial diseases. Although MMPs are temporally and spatially regulated during development, most mice with targeted deletions of MMPs develop

normally[97]. However, when challenged with specific pathological stimuli, many of these genetically altered mice respond differently from control mice, suggesting a clinical role for MMPs in many heart diseases. Ducharme et al. found a reduction in progressive ventricular dilation and collagen accumulation in infarcted hearts from MMP-9 deficient mice[14]. Heymans et al. also found a reduction in early myocardial rupture in MMP-9 deficient mice subjected to myocardial infarction[32]. The overall minimal phenotypes observed to date may be due to redundancy, safeguarding the host from untoward consequences of individual MMP mutations. In the future, generation of double and multiple MMP deficient mice may be required to unmask the full range of MMP function in the heart.

My results provide compelling evidence that MMP-2 acts as a mediator of cytokine-induced myocardial dysfunction. Several observations support this: (1) there was a rapid and marked increase of MMP-2 activity in perfusate, (2) this was followed by a decrease of MMP-2 and TIMP-4 in heart tissue following cytokine treatment, (3) Ro31-9790 and PD166793, inhibitors of MMP activity, prevented and a neutralizing MMP-2 antibody abolished the depression in mechanical function, and (4) the MMP-2 antibody prevented the loss of tissue MMP-2 activity. This latter effect of the antibody may have been through either intracellular or extracellular actions (or a combination of both). For example, it may have inhibited the enhanced secretion and/or activity of MMP-2 into the extracellular compartment. This would prevent "outside-in" cellular signals from MMP-2 acting upon membrane β_1 integrins receptors[83] (Figure 4.1). The MMP-2 antibody, however, did not affect the cytokine-induced increase in inducible NO

synthase activity, excluding the possibility that it nonspecifically inactivated cytokines or interfered with the expression of inducible NO synthase.

The mechanism of the association between MMPs and impaired contractile function is not clear. Lonn et al. showed that a decrease in collagen content occurred as early as at 30 minutes of referfusion following ischemia which was related to an increased activity of collagenase in isolated rat heart [49]. Hence, the release and increased activity of MMP-2 could be involved in the modification of the extracellular matrix and the associated myocardial dysfunction. Apart from the extracellular matrix, little is known about other possible targets of MMPs. The effect of MMP-2 in the control of Ca^{2+} pump activity particularly on intracellular Ca^{2+} concentration, may also be related to its effect on the recovery of mechanical function of the heart[87]. Inhibition of MMP-2 may alleviate the rise in intracellular Ca^{2+} concentration resulting in better recovery of cardiac function. Recently, an intracellular association of MMP-2 with the sarcolemma was found in hearts from patients with dilated cardiomyopathy[84]. This was accompanied by evidence that myosin heavy chain was susceptible to proteolytic cleavage by MMP-2 in vitro. Moreover, myosin heavy chain degradation products were also detected in cardiomyopathic hearts[84]. This indicates that contractile proteins may represent a molecular target for the detrimental actions of MMP-2 in the myocardium and that MMP-2 may have an intracellular locus of action. Indeed, this laboratory has recently shown that the contractile protein regulatory element troponin I is susceptible to rapid proteolytic cleavage by MMP-2 and that inhibition of MMP activity prevents troponin I

degradation while improving the recovery of mechanical function in ischemic–reperfused hearts[96]. Whether these contractile proteins are targets for MMPs in cytokine-induced myocardial depression is currently under investigation. Recently, several novel substrates and resulting biological activities of MMP-2 independent of its well-described activities on the extracellular matrix have been described. This includes roles for MMP-2 in the control of vascular tone[19], platelet aggregation[90], inflammation[54]and myocardial ischemic reperfusion injury[10], suggesting that this protease can have effects on a more rapid timescale and with both intracellular and extracellular loci of action (Figure 4.1).

Apart from MMP-2, MMP-9, another member of the gelatinases, may also play a role in tissue injury in inflammatory heart disease. MMP-9 is readily induced by pro-inflammatory cytokines and oncogene products and expressed in high levels by several types of inflammatory cells including neutrophils, macrophages, lymphocytes and eosinophils. Increased MMP-9 activity was found in infarcted heart tissue compared to normal myocardium[105]. Neonatal cardiac myocytes exposed to pro-inflammatory cytokines show enhanced MMP-9 activities[47]. Inhibition of MMP-9 by anti-MMP-9 antibody reduced infarct size in the brains of rats subjected to focal stroke[82]. Deletion of MMP-9 attenuated left ventricular enlargement and collagen accumulation after experimental myocardial infarction in mice[14]. Although much has been learned about MMP-9 since its identification, more work still remains to be done to further explore its role in pathogenesis.

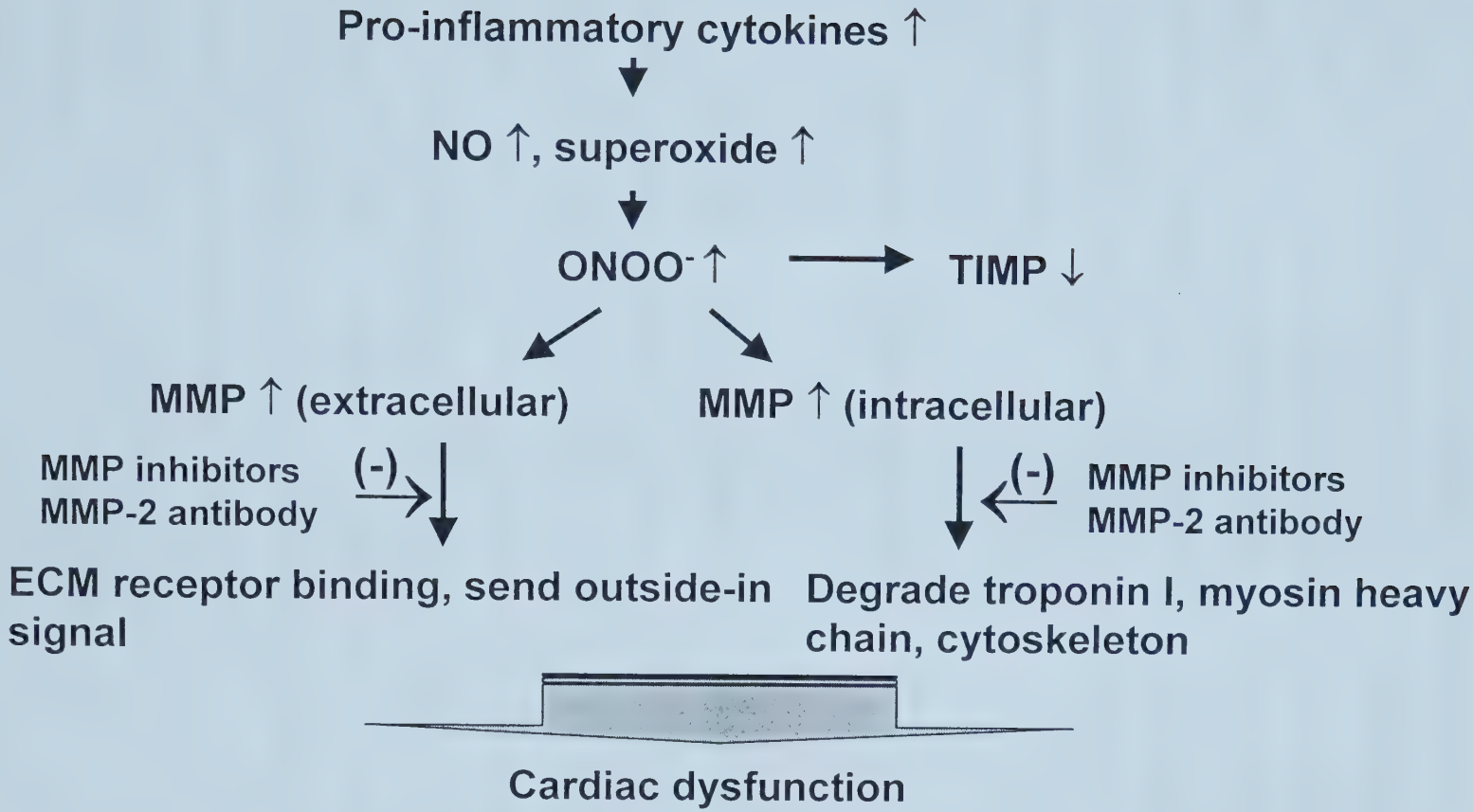

Figure 4.1 Schematic presentation of posssible mechanisms by which MMPs mediate cardiac dysfunction induced by pro-inflammatory cytokines. It has been shown that pro-inflammatory cytokines, via concerted increase of NO and superoxide, enhance the formation of their reaction product, $ONOO^-$. $ONOO^-$ may activate MMP both extracellularly and intracellularly as well as inhibit TIMP action. Intracellularly, the contractile protein regulatory element troponin I, myosin heavy chain, and the cytoskeleton may be susceptible to proteolytic cleavage by MMP-2. Inhibition of MMP activity prevents this degradation while improving the recovery of mechanical function. Extracellularly, inhibition of MMP may block the enhanced secretion and/or activity of MMP-2, thereby preventing "outside-in" cellular signals from MMP-2 acting upon membrane integrin receptors.

In summary, I have demonstrated that pro-inflammatory cytokines induced depression of mechanical function of the heart is mediated by MMP-2. Inhibition of MMP activity protects the heart from cytokine-induced myocardial injury, thus MMP-2 may be a viable target for the therapeutic intervention of inflammatory heart disease. Whether inhibition of MMPs will treat or prevent the development of cardiac dysfunction in diverse immune system-related cardiac diseases requires further studies.

A. Scavenging peroxynitrite study

In this study, I have demonstrated that the $ONOO^-$ decomposition catalyst, FeTPPs, prevents the decline in myocardial function induced by pro-inflammatory cytokines. I also showed that FeTPPs and its inactive form TPPs have MMP inhibitory effects in vitro, which could play a role in their beneficial effect.

Many studies suggest that NO itself is a cardioprotective and antioxidant molecule[85]. Under normal physiological conditions, low levels of NO production can cause coronary vasodilation, inhibit platelet aggregation, prevent neutrophil adhesion, modulate myocardial contractile function, inhibit mitochondrial O_2 consumption, and lower intracellular Ca^{2+} concentration via guanylate cyclase[62]. Under conditions of pro-inflammatory cytokines or bacterial endotoxin, the expression and activity of inducible NOS increases in cardiac myocytes, coronary vascular endothelium and endocardium to produce sustained and higher rates of NO biosynthesis. This resultant enhanced production of NO contributes in part to

depressed cardiac mechanical function, as it can only be partially blocked by the nonselective NO synthase inhibitor N^G-nitro-L-arginine methyl ester[94] or the selective iNOS inhibitor mercaptoethylguanidine[72].

In the clinic, the high mortality associated with systemic inflammatory response syndrome urges the development of pharmacological tools to prevent depressed cardiac contractile function. However, inhibition of NO production has not yet translated into viable therapeutic modalities. NOS inhibitors could not fully recover myocardial function and caused severe pulmonary hypertension and further depression of right ventricular function in human septic shock[2]. There are difficulties in appropriately titrating these drugs without losing the beneficial physiological functions of NO. Thus, downstream of NO need to be uncovered and exploited as therapeutic targets.

The toxicity of NO is markedly enhanced by its reaction with superoxide to form $ONOO^-$[4]. This laboratory has shown that the infusion of $ONOO^-$ into working rat hearts impairs cardiac contractile function by decreasing cardiac effiency[92]. Moreover, endogenous formation of $ONOO^-$ in the heart contributes to myocardial stunning in ischemia/reperfusion injury[110,119]. Ferdinandy et al. showed that $ONOO^-$ is a major contributor to cytokine-induced myocardial contractile failure[18]. Although some protective actions of $ONOO^-$ were found in myocardial ischemia-reperfusion injury in vivo, this was highly concentration-dependent and due to its reaction with free or protein-associated thiols to form nitrosothiols, and was lost at higher concentration[68,114]. Taken together, there is consensus that endogenous formed $ONOO^-$ contributes to myocardial injury.

Specific pharmacological targeting of $ONOO^-$ is an exciting new strategy to protect the heart from oxidant stress injury, especially since other antioxidant therapies with superoxide dismutase in canine hemorrhagic shock[38] or with ascorbate and α-tocoph-erol in septic shock patients[24] showed only limited beneficial effects on cardiac function.

FeTPPs, a peroxynitrite scavenger, catalyzes the isomerization of $ONOO^-$ to nitrate anion and thereby decreases its decomposition to highly reactive intermediates such as nitrogen dioxide and hydroxyl radicals[56]. It does not have any direct NO-scavenging effect[56]. FeTPPs did not affect the cytokine-induced increase in iNOS activity, excluding the possibility that FeTPPs nonspecifically inactivated cytokines and interfered with enzyme induction. FeTPPs protected against $ONOO^-$-induced cell death stimulated by cytokines or by its exogenous supply in macrophages and in mixed neuron-glia cultures. Some iron porphyrins related in structure to FeTPPs may also have a superoxide-scavenging effect[73], which could have contributed to its beneficial actions.

In addition to $ONOO^-$ scavenging effect, we also found both FeTPPs and TPPs have additional MMP inhibitory effect. In vitro, FeTPPs is a more potent MMP inhibitor than TPPs. It is possible that impurity of the commercially available TPPs may contain trace amount of FeTPPs. This may also contributed to FeTPPs beneficial effect and explain the intermediate protective effect of TPPs in cytokine-induced myocardial dysfunction. The mechanism is not clear yet.

Taken together, we need to do more experiments to explore the roles of $ONOO^-$ in pathological myocardial dysfunction. In the future, designing $ONOO^-$

scavenging drugs with additional MMP inhibitiory effect may provide a novel approach to protect the hearts from proinflammatory stress such as heart failure, cardiomyopathy, and viral, parasitic, and bacterial infections.

C. Limitations:

Several potential limitations exist in the use of isolated hearts to examine physiological consequences of cytokine exposure. Although the isolated heart perfusion model allows both functional, biochemical, and pharmacological studies of the heart including the coronary vasculature and the myocardium, there is a lack of interaction of the heart with the peripheral vasculature and other systems in the body. Caution is required in extrapolating the results from crystalloid-perfused hearts to those of blood-perfused hearts. Blood contains erythrocytes, leukocytes, platelets, and plasma, all of which have significant effect on heart function as well as on the effects of NO, $ONOO^-$, and MMPs (especially MMP-9). Inflammatory heart disease is associated with platelet and leukocyte activation, and the use of crystalloid perfusate precludes the study of myocardial depression due to platelet and leukocyte activation.

Moreover, I was not able to study a complete concentration-response curve in the analysis of potency and efficacy of some pharmacological agents (PD166793, Ro31-9790) because of the limited solubility in the perfusion buffer. Dimethyl sulfoxide, a vehicle for both PD166793 and Ro31-9790, is a potent antioxidant itself which has been shown to be beneficial to septic shock by

inhibiting NFκB and AP-1 activation[15]. Also, I was not able to detect the full array of potential MMPs and/or TIMPs because of limited detecting range of gelatin zymography. Other substrate zymography like casein zymography may apply to solve this problem. Meantime, I cannot identify the cellular source or the transport mechanism which result in the release of proMMP-2 and MMP-2 into coronary circulation from the heart.

Technically, zymography cannot differentiate whether the 72 kDa band represented a pure population of proMMP-2 or a mixed population of proMMP-2 and active MMP-2. Also, it does not provide information on the actual net proteolytic activity in the tissue because of the presence of TIMPs. In order to estimate net endogenous active MMP expression, in situ zymography need to be performed. The currently available inhibitors of MMP-2 are far from selective in their inhibition of MMP-2. There all share in common the ability to chelate zinc, which may also inhibit other zinc-containing enzymes such as superoxide dismutase. In addition to MMP inhibition, some MMP inhibitors such as doxycycline, minocycline may prevent pro-TNF-α processing to active from, therefore inhibit cytokine production[57,99]. Also, MMP inhibitors have been found to block iNOS activity and thus reduce iNOS expression[25]. These findings may provide other mechanisms for protective effect of MMP inhibitors.

D. Future directions:

For future studies, using a combination of drugs which reduce $ONOO^-$ formation and inhibit MMPs, may be more effective in improving cardiac function of inflammatory heart disease than the partial protection seen with either strategy alone. Given the limited improvement of cardiac function by MMP inhibitors, which could be related to the bioavailability to the cell, there is a need to develop effective MMP-2 inhibitors with better selectivity and bioavailability. Use of NOS inhibitors like N^G-monomethyl-L-arginine and superoxide dismutase may help to test the hypothesis that $ONOO^-$ activates proMMP-2 in isolated hearts subjected to pro-inflammatory cytokines. Since endogenous TIMPs are an important control point of MMP activity, future investigation into the function and regulation of TIMPs in inflammatory heart disease is also warranted. Experiments from several laboratories indicate that activation of MMPs is implicated in diverse cardiac diseases including ischemia, infarction, and heart failure in humans. In this regard, it will be important in my future studies to identify MMP activation in human inflammatory heart disease, as well as to develop new strategies for inhibition of MMP expression and activity.

E. Conclusions:

This study demonstrated that changes of MMP activity and TIMP expression may contribute to the pathological changes in pro-inflammatory cytokine-induced injury in the hearts. Pharmacological inhibition of MMP activity or scavenging peroxynitrite is able to improve the recovery of myocardial dysfunction subjected to pro-inflammatory cytokines. Thus, the concept of

controlling MMP expression and activity and scavenging peroxynitrite represent a new and exciting therapeutic target for treating inflammatory heart disease, heart failure, and cardiomyopathy.

CHAPTER V

REFERENCES

References

1. Arstall MA, Sawyer DB, Fukazawa R, Kelly RA. Cytokine-mediated apoptosis in cardiac myocytes: the role of inducible nitric oxide synthase induction and peroxynitrite generation. Circ Res. 1999;85:829-840.
2. Avontuur JA, Biewenga M, Buijk SL, Kanhai KJ, Bruining HA. Pulmonary hypertension and reduced cardiac output during inhibition of nitric oxide synthesis in human septic shock. 1998;9:451-454.
3. Baghelai K, Marktanner R, Dattilo JB, Dattilo MPM, Jakoi ER, Yager DR, Makhoul RG, Wechsler AS. Decreased expression of tissue inhibitor of metalloproteinase 1 in stunned myocardium. J Surg Res. 1998;77:35-39.
4. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA. 1990;87:1620-1624.
5. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. Am J Physiol. 1996;271:C1424-C1437.
6. Brown PD, Levy AT, Margulis IMK, Loitta LA, Stetler-Stevenson WG. Independent expression and cellular processing of Mr 72,000 type IV collagenase and interstitial collagenase in human tumorigenic cell lines. Cancer Res. 1990;50:6184-6190.

7. Brown PD, Kleiner DE, Unsworth EJ, Stetler-Stevenson WG. Cellular activation of the 72kDa type IV procollagenase/TIMP-2 complex. Kidney Int. 1993;43:163-70.

8. Carreras MC, Pargament GA, Catz SD, Podereso JJ, Boveris A. Kinetics of nitric oxide and hydrogen peroxide production and formation of peroxynitrite during the respiratory burst of human neutrophils. FEBS Letters. 1994;342:65-68.

9. Cheung PY, Danial H, Jong J, Schulz R. Thiols protect the inhibition of myocardial aconitase by peroxynitrite. Arch Biochem Biophys. 1998;350:104-108.

10. Cheung PY, Sawicki G, Wozniak M, Wang W, Radomski MW, Schulz R. Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. Circulation. 2000;101:1833-1839

11. Cleutjens JPM, Kandala JC, Guarda E, Guntaka RV, Weber KT. Regulation of collagen degradation in the rat myocardium after infarction. J Mol Cell Cardiol. 1995;27:1281-92.

12. Coker ML, Doscher MA, Thomas CV, Galis ZS, Spinale FG. Matrix metalloproteinase synthesis and expression in isolated LV myocyte preparations. AM J Physiol. 1999;277:H777-787.

13. de Belder AJ, Radomski MW, Why HJ, Richardson PJ, Martin JF. Myocardial calcium-independent nitric oxide synthase activity is present in dilated cardiomyopathy, myocarditis, and postpartum

cardiomyopathy but not in ischaemic or valvar heart disease. Br Heart J. 1995;74:426-30.

14. Ducharme A, Frantz S, Aikawa M, et al. Targeted deletion of MMP-9 attenuates left ventricular enlargement and collagen accumulation after experimental myocardial infarction. J Clin Invest. 2000;106:55-62.

15. Essani NA, Fisher MA, Jaeschke H. Inhibition NF-κB activation by dimethyl sulfoxide correlates with suppression of TNF-α formation, reduced ICAM-1 gene transcription, and protection against endotoxin-induced liver injury. Shock.1997;7:90-97.

16. Evans T, Carpenter A, Kinderman H. Evidence of increased nitric oxide production in patients with the sepsis syndrome. Circ Shock. 1993;41:71-81.

17. Ferdinandy P, Panas D, Schulz R. Peroxynitrite contributes to spontaneous loss of cardiac efficiency in isolated working rat hearts. Am J Physiol.1999;276:H1861-H1867.

18. Ferdinandy P, Danial H, Ambrus I, Rothery R, Schulz R. Peroxynitrite is a major contributor in cytokine-induced myocardial injury. Circ Res. 2000;87:241-247.

19. Fernandez-Patron C, Radomski MW, Davidge ST. Vascular matrix metalloproteinase-2 cleaves big endothelin-1 yielding a novel vasoconstrictor. Circ Res. 1999;85:906-911.

20. Fernandez-Patron C, Stewart KG, Zhang YL, Koivunen E, Radomski MW, Davidge ST. Vascular matrix metalloproteinase-2-dependent

cleavage of cacitonin gene-related peptide promotes vasoconstriction. Circ Res. 2000;87:670-676.

21. Ferry G, Lonchampt M, Pennel L, Nanteuil G, Canet E, Tucker GC. Activation of MMP-9 by neutrophil elastase in an in vivo model of acute lung injury. FEBS Lett 1997;402:111-115.

22. Fini ME, Cook JR, Mohan R, Brinckerhoff CE. Regulation of matrix metalloproteinase gene expression. Matrix metalloproteinases. San Diego: Academic Press, 1st, ed. 1998:299-356.

23. Frears ER, Zhang Z, Blake DR, O'Connell JP, Winyard PG. Inactivation of tissue inhibitor of metalloproteinase-1 by peroxynitrite. FEBS Lett. 1996;381:21-24.

24. Galley HF, Howdle PD, Walker BE, Webster NR. The effects of intravenous antioxidants in patients with septic shock. Free Radic Biol Med. 1997;23:768-774.

25. Golub LM, Lee HM, Ryan ME. Tetracyclines inhibit connective tissue breakdown by multiple non-antimicrobial mechanisms. Adv Dent Res. 1998;12:12-26.

26. Gomez DE, Alonso DE, Yoshiji H, Torgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological function. Eur J Cell Biol. 1997;74(2):111-122.

27. Greene J, Wang YE, Liu LA, Raymond CR, Shi YE. Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. J Biol Chem. 1996;271:30375-30380.

28. Grocott-Mason RM, Shah AM. Cardiac dysfunction in sepsis: new theories and clinical implications. Intens Care Med.1998;24:286-295.

29. Gross J, Lapiere CM. Collagenolytic activity in amphibian tissues: A tissue culture assay. Proc. Natl. Acad. Sci. USA. 1962;48:1014-1022.

30. Guzman NJ, Fang MZ, Tang SS, Ingelfinger JR, Gary LC. Autocrine inhibition of Na^+/K^+-ATPase by nitric oxide in mouse proximal tubule epithelial cells. J Clin Invest. 1995;95:2083-2088.

31. Hare JM, Colucci WS. Role of nitric oxide in the regulation of myocardial function. Prog Cardiovasc Dis. 1995;155-166.

32. Heymans S, Luttun A, Nuyens D, et al. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. Nat Med. 1999;5:1135-1142.

33. Hooper NM. Families of zinc metalloproteinases. FEBS Letters. 1994;354:1-6.

34. Huhtala P, Tuuttila A, Chow LY, Zohi J, Keski-Oja J, Tryggvason K. Complete structure of the human gene for 92 kDa type IV collagenase. J Biol Chem. 1991;266:16485-90.

35. Ischiropolous H, Zhu L, Beckman JS. Peroxynitrite formation from macrophage derives nitric oxide. Arch Biochem Biophys. 1992;298:446-451.

36. Ishida H, Ichimori K, Hirota Y, Fukahori M, Nakazawa H. Peroxynitrite-induced cardiac myocyte injury. Free Radic Biol Med. 1996;20:343-350.

37. Ishiyama S, Hiroe M, Nishikawa T, Abe S, Shimojo T, Ito H, Ozasa S, Yamakawa K, Matsuzaki M, Mohammed MU, Nakazawa H, Kasajima T, Marumo F. Nitric oxide contributes to the progression of myocardial damage in experimental autoimmune myocarditis in rats. Circulation.1997;95:489-496.

38. Kapoor R, Kalra J, Prasad K. Cardiac depression and cellular injury in hemorrhagic shock and reinfusion: role of free radicals. Mol Cell Biochem. 1997;76:291-301.

39. Kelly RA, Balligand JL, Smith TW. Nitric oxide and cardiac function. Circ Res. 1996;79:363-380.

40. Kikuchi K, Kadono T, Furue M, Tamaki K. Tissue inhibitor of metalloproteinase 1(TIMP) may be an autocrine growth factor in scleroderma fibroblasts. J Invest Dermatol. 1997;108:281-284.

41. Kim HE, Dalal SS, Young Erik, et al. Disruption of the myocardial extracelluar matrix leads to cardiac dysfunction. J Clin Invest. 2000;106:857-866.

42. Kooy NW, Lewis SJ, Royall JA, Ye YZ, Kelly DR, Beckman JS. Extensive tyrosine nitration in human myocardial inflammation: evidence for the presence of peroxynitrite. Crit Care Med. 1997;25:812-819.

43. Kooy NW, Royall JA. Agonist induced peroxynitrite production from endothelial cells. Arch Biochem Biophys. 1994;310:352-359.

44. Lafon-Cazal M, Culcasi M, Gaven F, Pietri S, Bockaert J. Nitric oxide, superoxide and peroxynitrite: putative mediators of NMDA-induced cell death in cellular granule cells. Neuropharmacology. 1993;32:1259-1266.

45. Langendorff O. Untersuchungen am uberlebenden Saugethierherzen. Arch Gesamte Physiol 1895;61:291.

46. Li YY, Feldman AM, Sun Y, McTiernan CF. Differential expression of tissue inhibitors of metalloproteinases in the failing human heart. Circulation. 1998;98:1728-1734.

47. Li YY, McTiernan CF, Feldman AM. Proinflammatory cytokines regulate tissue inhibitors of metalloproteinases and disintegrin metalloproteinase in cardiac cells. Cardiovasc Res. 1999;42:162-172.

48. Liu P, Hock CE, Nagele R, Wong PY. Formation of nitric oxide, superoxide, and peroxynitrite in myocardial ischemia-reperfusion injury in rats. Am J Physiol. 1997;272:H2327-H2336.

49. Lonn E, Factor SM, Van Hoeven KH, Wen WH, Zhao M, Dawood F, Liu P. Effects of oxygen free radicals and scavengers on the cardiac extracellular collagen matrix during ischemia-reperfusion. Can J Cardiol. 1994;10:203-213.

50. Lowenstein CJ, Hill S, Lafond-Walker A, Wu J, Allen G, Landavere M, Rose NR, Herskowitz, A. Nitric oxide inhibits viral replication in murine myocarditis. J Clin Invest. 1996;97:1837-1843.

51. Lo YC, Cruz TF. Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes. J Biol Chem. 1995;270:11727-30.

52. Maeda H, Okamoto T, Akaike T. Human matrix metalloproteinase activation by insults of bacterial infection involving proteases and free radicals. Biol. Chem. 1998;379:(2):193-200.

53. Mayers I, Salas E, Hurst T, Johnson D, Radomski MW. Increased nitric oxide synthase activity after canine cardiopulmonary bypass is suppressed by s-nitrosoglutathione. J Thorac Cardiovasc Surg. 1999;117(5):1009-1016.

54. McQuibban GA, Gong JH, Tam EM, McCulloch CA, Clark-Lewis I, Overall CM. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. Science. 2000;289:1202-1206.

55. Mihm MJ, Coyle CM, Schanbacher BL, Weinstein DM, Bauer JA. Peroxynitrite induced nitration and inactivation of myofibrillar creatine kinase in experimental heart failure. Cardiovasc Res. 2001 Mar;49(4):798-807.

56. Misko TP, Highkin MK, Veenhuizen AW, Mannning PT, Stern MK, Curri MG, Salvemini D. Characterization of the cytoprotective action of

peroxynitrite decomposition catalysts. J Biol Chem. 1998;273:15646-15653.

57. Mohler KM, Sleath PR, Fitzner JN, Cerretti DP, Alderson M, Kerwar SS, Torrance DR, Greenstreet T, Weerawarna K. Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. Nature. 1994;370(6486):218-20.

58. Moncada S, Higgs EA. The L-arginine-nitric oxide pathway. New Engl J Med. 1993;329:2002-2012.

59. Moreno JJ, Pryor WA. Inactivation of alpha 1-proteinase inhibitor by peorxynitrite. Chem Res Toxicol. 1992;5:425-431.

60. Moro MA, Darley-Usmar VM, Goodwin DA, Read NG, Zamora-Pino R, Feelisch M, Radomski MW, Moncada S. Paradoxical fate and biological action of peroxynitrite on human platelets. PNAS USA. 1994;91:6702-6706.

61. Moro MA, Darley-Usmar VM, Lizasoain I, Su Y, Knowles RG, Radomski MW, Moncada S. The formation of nitric oxide donors from peroxynitrite. Br J Pharmacol. 1995;116:1999-2004.

62. Moro MA, Russel RJ, Cellek S, et al. cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of soluble guanylate cyclase. PNAS USA 1996;93:1480-1485.

63. Murakami K, Kobayashi F, Ikegawa R. Metalloproteinase inhibitor prevents hepatic injury in endotoxemic mice. Eur J Pharma. 1998;341:105-110.

64. Murrell GA, Jang D, Williams R. Nitric oxide activates metalloproteinase enzymes in articular cartilage. Biochem Biophys Res Commun. 1995;206:15-21.

65. Nagase H. Activation mechanism of matrix metalloproteinases. Biol Chem. 1997;378:151-160.

66. Nathan C, Xie QW. Regulation of biosynthesis of nitric oxide. J Biol Chem. 1994;269:13725-13728.

67. Neely JR, Liebermeister H, Battersby EJ, Morgon HE. Effect of pressure development on oxygen consumption by isolated rat heart. Am J Physiol. 1967;212:804-814.

68. Nossuli TO, Hayward R, Jensen D, Scalia R, Lefer AM. Mechanisms of cardioprotection by peroxynitrite in myocardial ischemia and reperfusion injury. Am J Physiol. 1998;275:H509-519.

69. Okamoto T, Akaike T, Nagano T, Miyajima S, Suga M, Ando M, Ichimori K, Maeda H. Activation of human neutrophil procollagenase by nitrogen dioxide and peroxynitrite: a novel mechanism for procollagenase activation involving nitric oxide. Arch Biochem Biophys. 1997;342:261-274.

70. Owens MW, Milligan SA, Jourd'heuil D, Grisham MB. Effects of reactive metabolites of oxygen and nitrogen on gelatinase A activity. Am J Physiol. 1997;273:(2Pt1):L 445-450.

71. Oyama J, Shimokawa H, Momii H, Cheng X, Fukuyama N, Arai Y, Egashira K, Nakazawa H, Takeshita A. Role of nitric oxide and

peroxynitrite in the cytokine-induced sustained myocardial dysfunction in dogs in vivo. J Clin Invest.1998;101:2207-2214.

72. Panas D, Khadour FH, Szabo C, Schulz R. Pro-inflammatory cytokines depress cardiac efficiency by a nitric oxide-dependent mechanism. Am J Physiol. 1998;275:H1016-1023.

73. Patel M, Day BJ. Metalloporphyrin class of therapeutic catalytic antioxidants. Trends Pharmacol Sci. 1999;20:359-364.

74. Pelletier JP, Jovanovic D, Fernades JC, Manning P, Connor JR, Currie MG, Battista JA, Marel-Pelletier J. Reduced progression of experimental osteoarthritis in vivo by selective inhibition of inducible nitric oxide synthase. Arthritis & Rheumatism. 1998;41(7):1275-86.

75. Peterson JR, Tosenbury WS, et al. Matrix metalloproteinase inhibition blocks progression of heart failure. Circulation 1997;95(suppl I):I-520. Abstract.

76. Petros A, Lamb G, Leone A, Moncada S, Bennett D, Vallance P. Effects of a nitric oxide synthase inhibitor in human with septic shock. Cardiovasc Res. 1994;28:34-39.

77. Pryor W, Squadrito GL. The chemistry of peroxynitrite: from the reaction of nitric oxide and superoxide. Am J Physiol. 1995;L699-722.

78. Radomski A, Sawicki G, Olson DM, Radomski MW. The role of nitric oxide and metalloproteinases in the pathogenesis of hyperoxia-induced lung injury in newborn rats. Br J Pharmacol. 1998;125:1455-1462.

79. Radomski MW, Palmer RMJ, Moncada S. Characterization of the L-arginine: nitric oxide pathway in human platelets. Br J Pharmacol. 1990;101:325-328.

80. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS. Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. J Clin Invest. 1996;98:2572-2579.

81. Rohde LE, Ducharme A, Arroyo LH, Ailawa M, Sukhova GH, Lopez-Anaya A, McClure KF, Mitchell PG, Libby P, Lee RT. Matrix metalloproteinase inhibition attenuates early left ventricular enlargement after experimental myocardial infarction in mice. Circulation. 1999;99:3063-3070.

82. Romanic AM, Raymond RF, Arlenth AJ, Ohlstein EH, Barone FC. Matrix metalloproteinase expression increases after cerebral focal ischemia in rats. Inhibition of matrix metalloproteinase-9 reduces infart size. Stroke. 1998;29:1020-30.

83. Ross RS, Pham C, Shai SY, Goldhaber JI, Fenczik C, Glembotski CC, Ginsberg MH, Loftus JC. β1 integrins participate in the hypertrophic response of rat ventricular myocytes. Circ Res. 1998;82:1160-1172.

84. Rouet-Benzineb P, Buhler J-M, Dreyfus P, Delcourt A, Dorent R, Perennec J, Crozatier B, Harf A, Lafuma C. Altered balance between matrix metalloproteinases (MMP-2 and MMP-9) and their tissue

inhibitors in human dilated cardiomyopathy: potential role of MMP-9 in myosin-heavy chain degradation. Eur J Hear Failure. 1999;!:337-352.

85. Rubbo H, Radi R, Trujillos M et al. Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. J Biol Chem. 1994;269:26066-26075.

86. Salgo MG, Squadrito GL, Pryor WA. Peroxynitrite causes apoptosis in rat thymocytes. Biochem Biophys Res Commun. 1995;215:1111-1118.

87. Sarkadi B, Enyedi A, Gardos G. Conformational changes of the in situ red cell membranes calcium pump affect its proteolysis. Biochimica at Biophysica Acta 1987;899:129-133.

88. Sasaki K, Hattori T, Fujisawa. Nitric oxide mediates interleukin-1-induced gene expression of matrix metalloproteinases and basic fibroblast growth factor in cultured rabbit articular chondrocytes. J Biochem. 1998;123:431-439.

89. Sato H, Tkino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, Seiki M. A matrix metalloproteinase expressed on surface of invasive tumor cells. Nature. 1994;370:61-65.

90. Sawicki G, Salas E, Murat J, Miszta-Lane H, Radomski MW. Release of gelatinase A during platelet activation mediates aggregation. Nature. 1997;386:616-619.

91. Sawicki G, Sanders EJ, Murat J, Miszta-Lane H, Radomski MW. Localization and translocation of MMP-2 during aggregation of human platelets. Thromb Haemost. 1998;80:836-839.

92. Schulz R, Dodge KL, Lopaschuk GD, Clanachan AS. Peroxynitrite impairs cardiac contractile function by decreasing cardiac effiency. Am J Physiol. 1997;272:H1212-1219.

93. Schulz R, Nava E, Moncada S. Induction and potential biological relevance of a Ca^{2+}-independent nitric oxide synthase in the myocardium. British J Pharma. 1992;105:575-590.

94. Schulz R, Panas DL, Catena R, Moncada S, Olley PM, Lopaschuk GD. The role of nitric oxide in cardiac depression in induced by interleukin-1β and tumor necrosis factor-α. Br J Pharmacol. 1995;114:27-34.

95. Schulz R, Triggle CR. Role of NO in vascular smooth muscle and cardiac muscle function. Trends Phamacol Sci. 1994;15:255-259.

96. Schulze C, Wang W, Sawicki G, Schulz R. Degradation of troponin I by matrix metalloproteinase-2 contributes to acute ischemia-reperfusion injury of the heart. Circulation. 2000;102(suppl I):II-203. Abstract.

97. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Curr. Opinion in Cell Biol. 1998;10:602-608.

98. Smith JA, Radomski MW, Schulz R, Moncada S, Lewis MJ. Porcine ventricular endocardial cells in culture express the inducible form of nitric oxide synthase. Br J Pharmacol. 1993;108:1107-1110.

99. Solorzano CC, Ksontini R, Pruitt JH. A metalloproteinase inhibitor prevents processing of tumor necrosis factor alpha and abrogates endotoxin induced lethality. Shock. 1997;7:427-431.

100. Spinale FG, Coker ML, Thomas CV, Walker JD, Mukherjee R, Hebbar L. Time-dependent changes in matrix metalloproteinase activity and expression during the progression of congestive heart failure: relation to ventricular and myocyte function. Circ Res. 1998;82:482-495.

101. Titheradge MA. Nitric oxide in septic shock. Bioch Biophy Acta. 1999;1411:437-455.

102. Tohse N, Sperelakis N. cGMP inhibits the activity of single calcium channels in embryonic chick heart cells. Circ Res. 1991;69:325-331.

103. Trachtman H, Futterweit S, Garg P, et al. Nitric oxide stimulates the activity of a 72 kDa neutral matrix metalloproteinase in cultured rat mesangial cells. Biochem. Biophy. Res. Commun. 1996;218:704-708.

104. Tummalapalli CM, Heath BJ, Tyagi SC. Tissue inhibitor of metalloproteinase-4 instigates apoptosis in transformed cardiac fibroblasts. J Cell Biochem. 2001;80:512-521.

105. Tyagi SC, Campbell SE, Reddy HK, et al. Matrix metalloproteinase activity expression in infarcted, noninfarcted, and dilated cardiomyopathic human hearts. Mol Cell Biochem. 1996;155:13-21.

106. Ungureanu-Longrois D, Balligand JL, Kelly RA, Smith TW. Myocardial contractile dysfunction in the systemic inflammatory response syndrome: role of a cytokine-inducible nitric oxide synthase in cardiac myocytes. J Mol Cell Cardiol. 1995;27:155-167.

107. Ungureanu-Longrois D, Balligand JL, Okada I, Simmons WW, Kobzik L, Lowenstein CJ, Kunkel SL, Michel T, Kelly RA, Smith TW.

Contractile reponsiveness of ventricular myocytes to isoproterenol is regulated by induction of nitric oxide synthase activity in cardiac microvascular endothelial cells in heterotypic primary culture. Circ Res. 1995;77:486-493.

108. Van Wart HE, Birkedal-Hansen H. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. PNAS USA. 1990;87:5578-5582.

109. Villa LM, Salas E, Darley-Usmar VM, Radomski MW, Moncada S. Peroxynitrite induces both vasodilation and impaired vascular relaxation in the isolated perfused rat heart. PNAS USA. 1994;91:12383-12387.

110. Wang P, Zweier JL. Measurement of nitric oxide and peroxynitrite generation in the postischemic heart. Evidence for peroxynitrite-mediated reperfusion injury. J Biol Chem 1996;271:29223-29230.

111. Wick M, Burger C, Brusselbach S, Lucibello FC, Muller R. A novel member of human tissue inhibitor of metalloproteinases (TIMP) gene family is regulated during G1 progression, mitogenic stimulation, differentiation and senescence. J Biol Chem. 1994;269:18953-18960.

112. Wildhirt SM, Dudek RR, Suzuki H, Pinto V, Narayan KS, Bing RJ. Immunohistochemistry in the identification of nitric oxide synthase isoenzyme in myocardial infarction. Cardiovas Res. 1995;29:526-531.

113. Wilhelm SM, Collier IV, Marmer BL, Eizen AZ, Marmer BL, Grant GA, Selter JL. Kronberger A, He C, Bauer E, Goldberg GI. H-ras oncogene transformed human branchial epithelial cells secrete a single metalloprotease capable of degrading basement collagen. J Biol Chem. 1988;263:6579-87.

114. Wink DA, Hanbauer I, Krishna MC, DeGraff W, Gamson J, Mitchell JB. Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. Proc Natl Acad Sci USA.1993;90:9813-9817.

115. Woessner JFJ. The matrix metalloproteinase family. Matrix metalloproteinases. San Diego: Academic Press;1998:1-14.

116. Wu I, Moses MA. Molecular cloning and expression and analysis of the cDNA encoding rat tissue inhibitor of metalloproteinase-4. Matrix Biol. 1998;16:339-342.

117. Xie YW, Wolin MS. Role of nitric oxide and its interaction with superoxide in the suppression of cardiac muscle mitochondrial respiration. Involvement in response to hypoxia/reoxygenation. Circulation.1996;94:2580-2586.

118. Yang X, Chowdhury N, Cai B, Brett J, Marboe C, Sciacca RR, Michler RE, Cannon PJ. Induction of myocardial nitric oxide synthase by cardiac allograft rejection. J Clin Invest. 1994;94:714-721.

119. Yasmin W, Strynadka KD, Schulz R. Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. Cardiovasc Res. 1997;33:422-32.

120. Yu AE, Murphy AN, Stetler-Stevenson WG. 72-kDa gelatinase (gelatinase A): structure, activation, regulation, and substrate specificity. Matrix metalloproteinases. San Diego: Academic Press;1998;

121. Zucker S, Drew M, Conner C, Foda H, Declerck Y, Langley K, Bahov W, Docherty A, Cao J. Tissue inhibitor of metalloproteinase-2 (TIMP-2) binds to the catalytic domain of the cell surface receptor, membrane type-1 matrix metalloproteinase 1 (MT1-MMP). J Biol Chem. 1998;273:1216-1222.